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A Continuous Flow System Using Fish and Amphibian Eggs for Bioassay Determinations on Embryonic Mortality and Teratogenesis

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A CONTINUOUS FLOW SYSTEM USING FISH AND AMPHIBIAN EGGS FOR BIOASSAY DETERMINATIONS ON EMBRYONIC MORTALITY AND TERATOGENESIS

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A procedure is described for continuous flow bioassays on sensitive developmental stages of fish and other aquatic species. Environmental toxicants may be screened for teratogenic and other embryopathic effects (e.g., mortality, locomotor impairment) on eggs, embryos, and early juvenile stages. Toxicant concentration may be regulated down to 1.0-0.1 ppb for an exposure period extending from fertilization through 4-7 days posthatching. Bioassays may be performed with either natural or synthetic water, and standard physicochemical parameters (e.g., temperature, water hardness, pH) can be manipulated independently to determine effects on toxicity The procedure is suitable for use with a wide range of fish and amphibian species, including rainbow trout, channel catfish, largemouth bass, leopard frog and others.

Concerning the toxic effects of many environmental trace contaminants, embryonic and early juvenile stages constitute the critical "sensitive links" in the life cycles of many aquatic animal species. Reproductive potential of acquatic animals may be severly restricted or abolished by trace levels of toxicants which are harmless or sublethal to most adult organisms.

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BY

Wesley J. Birge
Jeffrey A. Black

EPA Contract No. 68-01-4321

EPA Project Officer
I. Eugene Wallen, Ph.D.

For

U. S. Environmental Protection Agency

Office of Toxic Substances
4th and M Streets, S. W.
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INTRODUCTION

It is becoming increasingly apparent that, concerning the toxic effects of many environmental trace contaminants, embryonic and early juvenile stages constitute the critical "sensitive links" in the life cycles of many animal species (1, 24). It appears plausible that reproductive potential of natural animal populations may be severely restricted or abolished by trace levels of toxicants which are harmless or sublethal to most adult organisms. Accordingly, protective environmental standards and pollution abatement policies, which have been based to a considerable extent on tolerances of adult animals, may not provide adequate protection for embryonic development and reproduction of many species.

Numerous organic toxicants are known to inhibit animal reproduction (1-18). For example, Bowes et al. (16) have found high levels of DDE and PCB in avian eggs, with PCB reaching egg concentrations of 300 ppm wet weight, and 3700 ppm in lipid fraction. These occurrences were correlated with embryonic death and reduced hatchability.

Also, PCB has been shown to adversely affect reproduction of fish and certain invertebrates. In continuous flow bioassays, exposure to 1.3 ppm Aroclor 1254 for three weeks produced 50% reproductive impairment in cultures of Daphnia magna (18), and a concentration of 0.32 ppb reduced survival of early fry of the sheepshead minnow to 62%, compared with 89% for controls (3). Nebeker et al. (5) recently investigated the effects of several PCB compounds on survival and reproduction of the fathead minnow. Aroclor 1254 at 4.6 ppb produced 100% mortality of eggs, and 0.52 ppb resulted in 59-67% hatchability, compared to 71-76% in control populations. Early fry and embryos were the most sensitive stages in this chronic life cycle study. Jensen et al. (6) reported that PCB residues of 0.4-1.9 ppm in salmon eggs resulted in mortality ranging from 16-100%. As the accumulation of PCB in fish eggs and tissues may be 75,000-200,000 times the concentration found in water (1, 19), environmental levels as low as 2-20 parts per trillion may inhibit egg hatchability in salmonid fishes.

Though generally less toxic than PCB compounds, DDT is known to concentrate in fish eggs and sperm, affecting hatchability and survival of fry (1, 7-9, 11-13). Egg concentrations of 0.4 to 4.75 ppm or more produce high levels of embryonic mortality in trout and salmon (1, 8, 9), and treatment of early salmon embryos with 50-100 ppb DDT retards behavioral development and impairs balance (12).

Furthermore, the exposure of adult flounder to DDT at a sublethal concentration of 2 ppb reportedly produces appreciable frequencies of anomalies among resulting offspring (11).

Various other chlorinated hydrocarbons, including 2,4-D, dieldrin and endrin are known to produce mortality of fish eggs and fry (10, 11, 20, 21). In static tests with bluegill, Hiltibran (10) proposed a threshold for developmental stages of approximately 2 ppm 2,4-D, and in chronic bioassays on the sailfin molly, 0.75 ppb dieldrin was found to reduce growth rate and reproduction (22). Working with endrin, Johnson (23) reported sublethal exposure to adult fish which resulted in egg concentrations sufficient to produce mortality of developing fry. Though many organic toxicants remain to be investigated, it is evident that animal reproduction is one of the principal target sites of chlorinated hydrocarbons and numerous other organic pollutants.

Numerous inorganic toxicants also exert adverse effects on animal reproduction, particularly aquatic species. Eggs, embryos and/or early juvenile stages of fish are substantially more susceptible than adults to such metals as cadmium, copper, mercury and zinc (24-30). For example, continuous flow treatment of rainbow trout eggs with 0.1 ppb inorganic mercury produced 100% embryonic mortality in 10-14 days (39). In the same investigation, trout and channel catfish embryos treated for 5 days at 0.1-3.0 ppb mercury were found to accumulate tissue mercury at 500-2000 times the exposure levels. In addition, frog and chick embryos are up to 1000-10,000 times more sensitive to mercury than are adult stages (24, 31-34).

Bioassays with boron compounds provide further evidence that developmental stages are appreciably more sensitive to environmental toxicants than are adult organisms. Working with the adult mosquito fish (Gambusia affinis), Wallen et al. (35) reported 96-hr TL_m values of 3600 and 5600 mg/l for sodium borate and boric acid, respectively. Using the figure given for boric acid, this amounts to an approximate dosage of 980 ppm boron. Turnbull et al. (36) determined a 24-hr TL_m of 15,000 mg/l for bluegill treated with boron triflouride administered at 20^0 C in Philadelphia tap water, and a 48-hr LD_{50} of 339 ppm boron has been reported for 15-month old rainbow trout (37, 38). In addition, Sprague (37) has given safe limits of 30 ppm and 33 ppm for largemouth bass and bluegill, respectively.

By comparison, low concentrations of boron produce high frequencies of mortality and teratogenesis in populations of fish, amphibian and avian embryos (40-45). Using continuous flow treatment from fertilization through 4 days posthatching, we obtained boron threshold (LC_1) values of 0.001-0.1 ppm, 0.2-1.4 ppm and 0.2-5.5 ppm for developmental stages of the rainbow trout, goldfish, and channel catfish treated with boric acid or borax at water hardness levels of 50-200 ppm $CaCO_3$ (45). Depending on test conditions, boron LC_{50} values for developmental stages ranged from 27-100 ppm for rainbow trout, 46-75 ppm for goldfish, and 22-155 ppm for catfish. Concerning avian species, which share a number of developmental characteristics with piscine forms (e.g., megalecithal eggs, meroblastic cleavage, primitive streak gastrulation), embryonic stages also are impaired by low dosages of boron compounds (43-45). Combining frequencies of embryonic mortality and teratogenesis in chick embryos treated with boric acid and borax, we obtained boron threshold and LC_{50} values of 0.01 ppm and 0.5-1.0 ppm, respectively. In contrast to the high boron sensitivity of chick embryos, laying hens have been found to tolerate a daily ration containing 0.5% boric acid (875 ppm boron). Though egg laying ceased after 6 days of exposure, normal egg production resumed within 2 weeks after treatment was discontinued (46).

Operating upon the premise that environmental quality standards for trace contaminants should be established at levels which are safe for the most susceptible stages in the life cycles of living organisms, consistent with the recommendations of the NTAC, NAS-NAE Committee on Water Quality Criteria, and others (1, 31, 47-50), we contend that sensitive developmental stages should be included among the test organisms used for the bioassay analysis of environmental pollutants.

Under support from the Department of Interior (Office of Water Research and Technology), Environmental Protection Agency (Office of Toxic Substances) and the National Science Foundation (RANN), we have developed a series of bioassay and bioindicator procedures for use in evaluating the effects of environmental toxicants on eggs, embryos and early juvenile stages of fish, amphibian and avian species. These test systems include a yolk sac injection technique for the chick embryos (33, 34), as well as static rapid-scan (24, 31, 51), continuous flow, and full life-cycle bioassays for aquatic vertebrates (39). Attention here will be restricted primarily to a continuous flow procedure for use with developmental and early juvenile stages of fish and amphibians, where exposure is maintained continuously from fertilization through 4-7 days posthatching. Resulting dose/

response data may be used to define tolerances of sensitive life cycle stages (e.g., mortality, embryonic anomalies, behavioral impairments) or to screen compounds for teratogenic properties.

ADVANTAGES OF FISH AND AMPHIBIAN EMBRYOS AS BIOASSAY TEST ORGANISMS

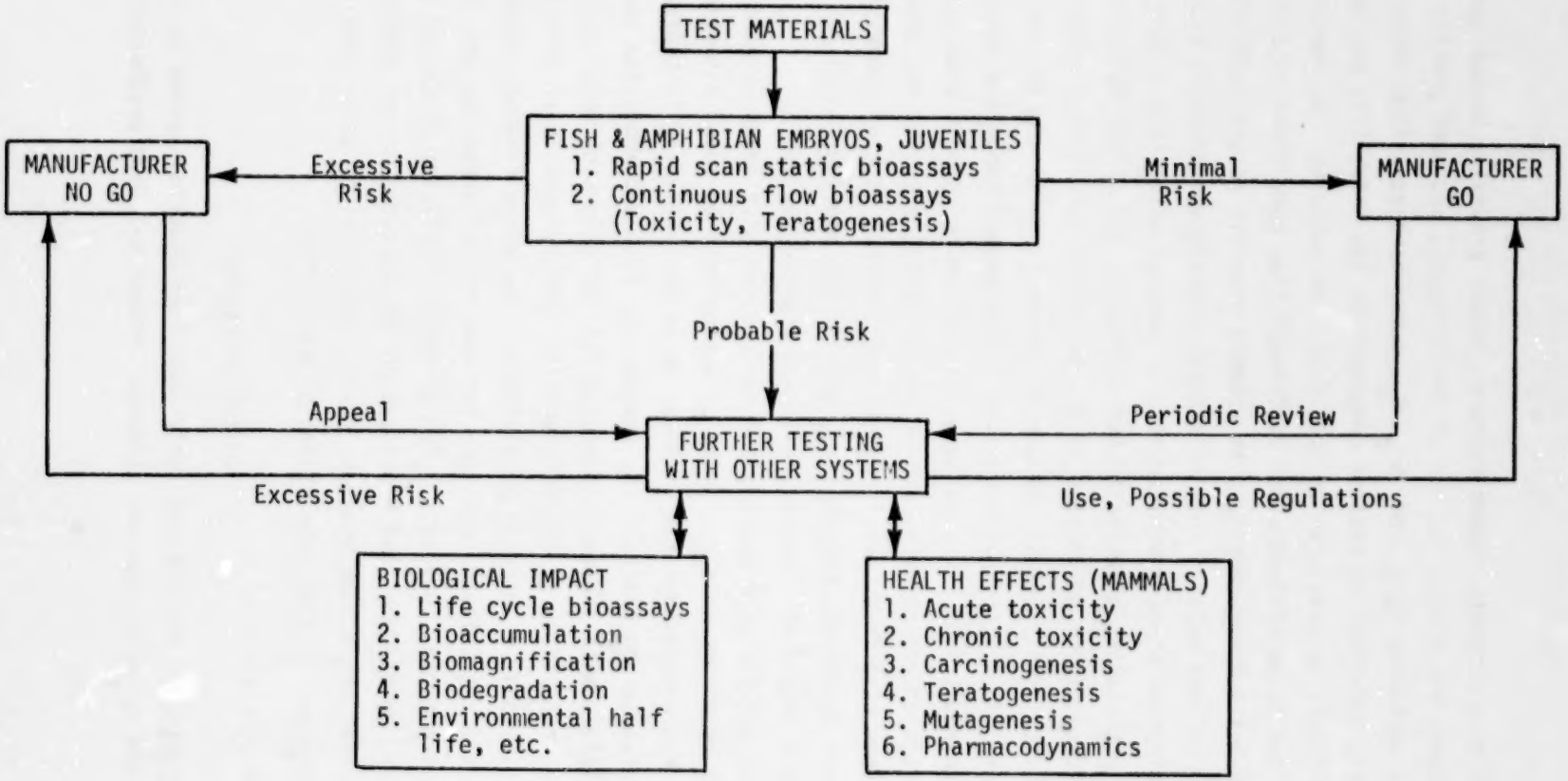
Developmental stages present a broad spectrum of target sites for the toxic action(s) of trace contaminants, including the biochemical and physiological mechanisms associated with fertilization, genic action, cell proliferation and growth, cellular differentiation, basic metabolism and systemic functions, as well as the hatching process and the initial accommodation to a free-living existence. As a consequence, bioassay screening with developmental stages is unlikely to produce "false negative" results, providing exposure to selected toxicants is maintained continuously from fertilization through 4-7 days posthatching.

Fish and amphibian developmental stages also display high levels of sensitivity to environmental contaminants. Concerning more toxic elements or compounds (e.g., Hg, Cd, chlorinated hydrocarbons), developmental stages of trout or other sensitive species usually exhibit irreversible test responses, including mortality, teratogenesis or locomotor impairment, at ppb to ppt exposure levels. The sensitivity of such bioassays often exceeds analytical detection limits. Less toxic substances (e.g., boron compounds) generally produce test responses at ppm to ppb concentrations. With respect to metallic toxicants (e.g., As, Hg, Pb, Zn), we usually detect mortality and/or teratogenesis of piscine developmental stages at exposure levels below those which initiate avoidance or other behavioral responses in adult fish (39, 45, 52-54). As they exhibit both high sensitivity and clearly detectable test responses, developmental stages are especially suitable for short-term bioassay studies. The schematic in Figure 1 indicates a possible means of using aquatic embryos and larvae for bioassay screening purposes.

Piscine developmental stages also possess a number of other advantages as test organisms. They are less subject than adult fish to physiological or behavioral variables induced by crowding or other test conditions. Also, they require no feeding and excrete minimal waste products, exerting far less burden on culture water than adult organisms. Test populations of 100-200 fish eggs can

FIGURE 1

AQUATIC EMBRYOS AND LARVAE AS SENSITIVE, BROAD SPECTRUM TEST ORGANISMS FOR BIOASSAY SCREENING OF ENVIRONMENTAL POLLUTANTS



be maintained in a compact, closed culture system, providing better control of exposure level and mixing pattern of the toxicant, and more precise regulation of other test variables (e.g., pH, NH_3, O_2, water hardness, flow rate). This is particularly important in testing compounds of low solubility or high volatility, which are difficult to stabilize in open fish tank cultures. As generous samples of fish eggs can be maintained under continuous flow conditions in culture chambers of 0.5-1.0 liter capacity, it becomes feasible to use synthetic or reconstituted culture water. This eliminates significant problems with background contamination found in many sources of natural water (e.g., heavy metals, chloramines, phthalates, chlorinated hydrocarbons), and allows formulation of desired water quality parameters (e.g., pH, total alkalinity, water hardness).

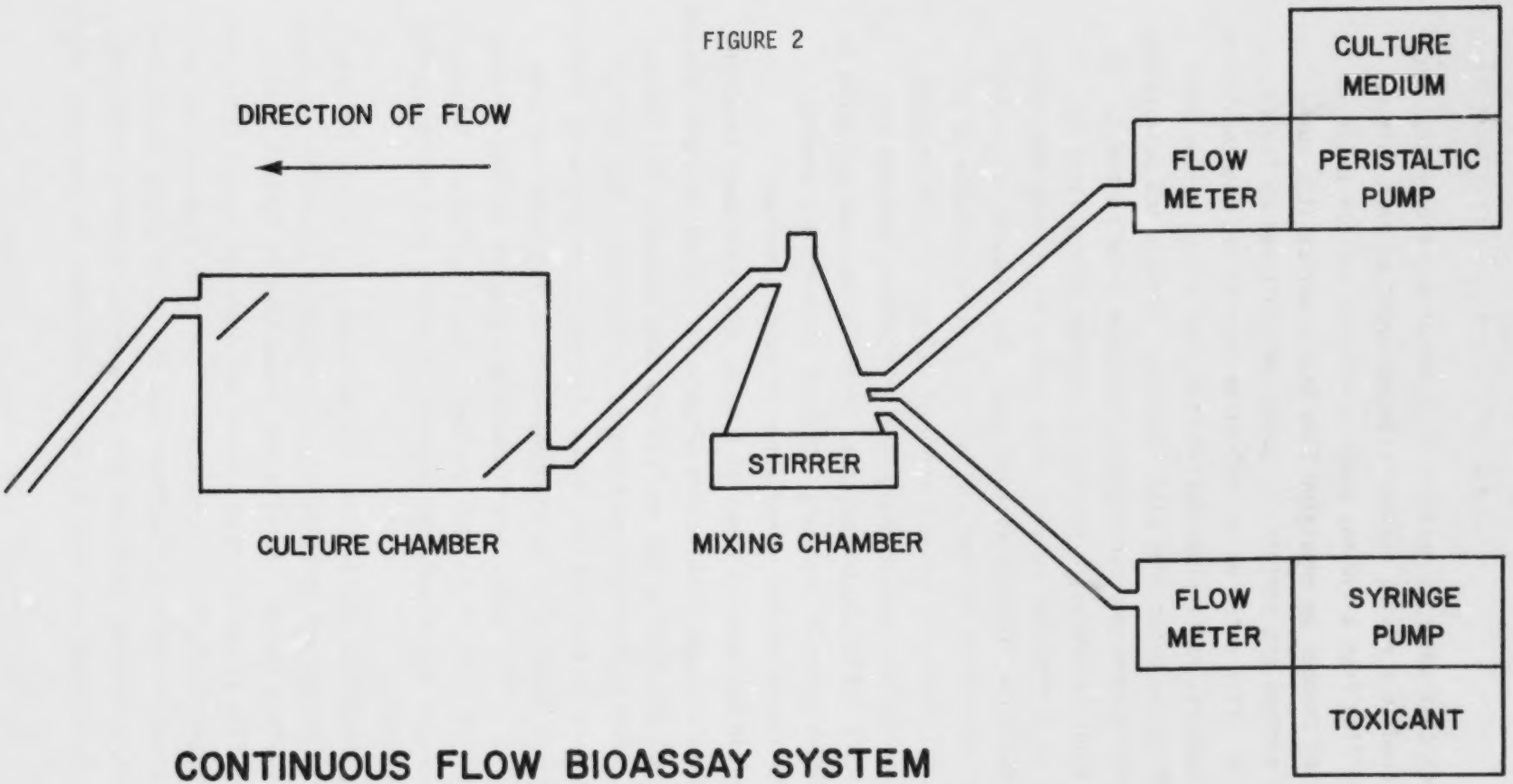
In egg hatchability tests, the use of synthetic culture water and the omission of feeding preclude the two major sources of background contamination which plague bioassays with adult fish. For example, in performing chronic bioassays with 0.1-1.0 ppb cadmium and mercury, one of our principal problems has been to secure contaminant-free fish food. We have analyzed numerous lots of commercial food which contained 10,000-20,000 ppb lead, 500-700 ppb cadmium, 20-30 ppb mercury, and appreciable levels of other metallic and organic toxicants.

In recent studies with mercury and cadmium, we have found threshold values obtained with trout egg cultures equal or approximate to those determined in chronic life cycle bioassays (39). Also, in an evaluation of 11 Central Kentucky streams and rivers, egg hatchability indices for fish and amphibian species correlated closely with independent ecological indicators of water quality (51; Table 19). In particular, reductions in egg hatchability closely paralleled decreases in density and diversity of piscine populations. The above findings further emphasized the value of the egg culture technique for the initial screening and characterization of environmental toxicants. Due to their simplicity of design and economy of maintenance, continuous flow bioassays on egg cultures may be performed in many laboratories which lack adequate space, facilities or water supply necessary for maintaining and testing populations of adult fish.

BIOASSAY PROCEDURES

Bioassay system. Our present continuous flow bioassay system is illustrated in Figures 2 and 3. Fish eggs are cultured through 4-7 days posthatching in pyrex

FIGURE 2



CONTINUOUS FLOW BIOASSAY SYSTEM

Peristaltic pump delivers culture water to mixing chamber at a prescribed flow rate. Toxicant is administered to the mixing chamber by graduated flow from a syringe pump, providing toxicant/culture water mixing ratios down to 1:1000 or less. The final mixture, agitated with a teflon-coated stirring bar, is pumped through the egg culture chamber under positive pressure. Mixing baffles in the culture chamber facilitate uniform flow. High resolution liquid flow meters are used to determine flow rates from syringe and peristaltic pumps.

chambers through which test water is perfused at a prescribed flow rate. The toxicant is administered to a mixing chamber situated ahead of each culture dish, using graduated flow from a syringe pump. Synthetic culture water is delivered to the mixing chamber by regulated flow from a peristaltic pump. Flow rates from both syringe and peristaltic pumps are monitored by liquid flow meters (Figure 3). Flow rates up to 2000 ml/hr or more are obtainable for 300-500 ml culture chambers, and syringe/peristaltic pump mixing ratios down to 1:1000 or 1:10,000 can be maintained with accuracy. Syringe and peristaltic pumps are provided with calibrated, continuously variable speed controls. The concentration of toxicant delivered to the mixing chamber is regulated by adjusting the mixing ratio from the pumping units and/or by varying the concentration of toxicant delivered from the syringe pump. For example, a toxicant at 0.1 ppm can be delivered from the syringe pump to a mixing chamber at 0.3 ml/hr, and diluted to a final test concentration of 0.1 ppb by culture water metered through the peristaltic pump at 300 ml/hr. Solutions from the two channels are mixed with a teflon-coated magnetic stirring bar, and delivered to the culture chamber under positive pressure. Culture chambers are provided with mixing baffles to insure uniform circulation of culture medium.

We normally maintain cultures in banks of 6 to 8, with each bank involving a single syringe/peristaltic pump combination (Figure 3). Using syringes selected for equal stroke volume, and fitting the peristaltic pump channels with tubing of matched diameters, highly consistent performance is obtained. Two control populations are maintained in each culture bank. Presently, we are using Brinkmann (model 131900) and Gilson (model HP8) multichannel peristaltic pumps and Sage syringe pumps (model 355). The latter are fitted with modified syringe holders (Figure 3.3), and operated using 10 ml double-ground, glass syringes. Tuberculin 1 ml or microliter syringes may be used for toxicant delivery when mixing ratios above 1:1000 prove necessary.

We have used methylene blue dye to check the accuracy of a 1:1000 syringe/peristaltic pump mixing ratio, and to allow visual inspection of mixing and flow patterns through the culture system. Results are summarized in Table 1. Also, the reproducibility of flow rates, dilution ratios and exposure levels for several boron bioassays is treated in Table 2. Accuracy of flow rates from syringe and peristaltic pumps is given in Table 5. We have used the above system extensively for bioassay measurements on boron compounds and on numerous metallic toxicants, using treatment levels down to 0.1 ppb. Monitoring of culture water for toxicant levels

TABLE 1

PERFORMANCE OF THE CONTINUOUS FLOW SYSTEM

Reproducibility of 1:1000 Mixing Ratio for an Aqueous Solution of Methylene Blue Dye (1000 µg/ml) Delivered at 0.3 ml/hr by a Sage Syringe Pump to Culture Chambers Receiving 300 ml/hr of Culture Water Provided by Gilson Peristaltic Pump[1]

CULTURE CHAMBER NO.	ACTUAL DYE CONCENTRATION[2,3,4] (µg/ml)
1	1.00 ±0.03
2	0.98 ±0.03
3	0.99 ±0.03
4	1.00 ±0.01
5	0.99 ±0.03
6	0.96 ±0.02

[1]The pumping rates stated above were set to give a final, theoretical dye concentration of 1 µg/ml culture water. Dye concentrations were determined spectrophotometrically (Cary model 17 recording spectrophotometer).

[2]Each concentration given as the mean value ± standard error for 20 measurements taken at regular intervals over a seven day period of continuous operation.

[3]A single Sage syringe pump (model 355) was used to operate all six syringes, and one multichannel Gilson peristaltic pump (model HP8) provided 6 separate supply lines for culture water.

[4]Sections of tygon tubing used for the different peristaltic pump channels were selected for matching diameters. Six double ground, 10 cc syringes were mounted on the syringe pump, using a modified syringe holder.

TABLE 2

SYRINGE PUMP (BORON) AND PERISTALTIC PUMP (CULTURE WATER) FLOW RATES WITH RESULTING DILUTION RATIOS OBTAINED IN TREATMENT OF TROUT EMBRYOS AT 1 AND 10 PPB BORON[1]

TROUT BIOASSAYS		SYRINGE PUMP (BORON)			PERISTALTIC PUMP (CULTURE WATER)		FINAL BORON CONCENTRATION[2] (µg/l)
COMPOUND/HARDNESS	NOMINAL BORON LEVEL (µg/l)	FLOW (µl/hr)	BORON (µg/l) THEORETICAL	BORON (µg/l) ACTUAL	FLOW (ml/hr)	BORON DILUTION RATIO	
BORIC ACID							
50 ppm $CaCO_3$	1.0	330 ± 3	600	594 ± 4	198 ± 2.4	600 ± 7	0.99
	10.0	330 ± 3	6000	6050 ± 90	198 ± 4.8	600 ± 15	10.08
200 ppm $CaCO_3$	1.0	318 ± 7	600	614 ± 4	183 ± 4.0	575 ± 13	1.07
	10.0	306 ± 1	6000	6130 ± 60	192 ± 3.7	627 ± 8	9.78
BORAX							
50 ppm $CaCO_3$	1.0	318 ± 4	600	588 ± 11	188 ± 2.9	591 ± 14	0.99
	10.0	325 ± 3	6000	5930 ± 80	190 ± 2.8	585 ± 11	10.14
200 ppm $CaCO_3$	1.0	320 ± 1	600	594 ± 4	194 ± 1.9	606 ± 7	0.98
	10.0	320 ± 1	6000	5870 ± 70	197 ± 1.3	616 ± 5	9.53

[1]Mean values ± standard errors, where n was 10 for syringe pump boron analyses and 20 for all other determinations.

[2]Final boron concentration delivered to the culture chamber was determined as actual syringe pump boron concentration/boron dilution ratio. The latter was calculated as peristaltic pump flow rate/syringe pump flow rate.

has shown good reproducibility (Tables 2, 6-8).

Modifications of present bioassay system. The above system may be modified in several important respects in order to accommodate testing of organic chemicals of low solubility or high volatility.

A closed culture chamber may be used to eliminate the air/water interface, minimizing volatility as a test variable. Such culture chambers may be constructed from 3" pyrex pipe provided with clamp-locking, teflon O-ring joints. Using standard glass blowing techniques, the glass pipe should be cut and sealed to give a capacity of 0.5 liter, and to position the O-ring-sealed joint as close as possible to the "top" of the culture dish. Access to the test organisms may be obtained by opening the watertight joint and removing the chamber cover. An outlet port should be annealed to the cover, with an inlet port positioned near the bottom of the chamber. The culture chamber may be divided by a teflon screen, positioned horizontally at mid-depth. Test eggs and embryos should be maintained above the screen, while a glass or teflon-coated, magnetic stirring bar may be used below the screen to provide moderate agitation of the culture water.

The second principal modification concerns the mixing chamber shown in Figure 2. This unit should be equipped with a high capacity variable speed stirring mechanism, suitable to provide a uniform suspension of insoluble toxicant in culture water, and flow rate through the culture system should be adjusted to minimize partitioning of the toxicant in the egg chamber. Some experimentation may be required to develop an adequate mixing technique which will fulfill bioassay requirements,without excessive foaming or other conditions unsuitable to the design of the continuous flow procedure. Mechanical mixing, if properly adapted to the system, may eliminate the need for carrier solvents or emulsifiers which introduce undesirable test variables. We are now in the process of testing and perfecting the above culture modifications, performing bioassays with a series of organic toxicants which vary in solubility and volatility.

Culture water. We have given considerable attention to the development of a synthetic culture water suitable for bioassays. Synthetic water provides more stable test conditions than natural water, as the latter is subject to substantial geographic and seasonal fluctuations in composition (e.g., total dissolved solids, hardness, pH). Also, problems with background contaminants are minimized when prepared water is used for culture purposes. However, it is essential to use a formulation for synthetic water which gives chemical and physical characteristics similar to natural water.

In the event that an ample supply of natural water of high quality is not available, the synthetic water described below may be used. This formulation has been used extensively during the past 2 years, and is known to give toxicity responses with metals (e.g., Cd, Cu, Hg, Zn) which are closely comparable to results obtained using natural water of similar composition. Also, we have performed hatchability tests with 4 species of fish and 5 species of amphibians, comparing our synthetic culture water to natural water from Steele's Run, a local stream (limestone bed) which supports a healthy aquatic ecosystem. Frequencies of hatchability were indistinguishable for the two water sources, averaging 92% for fish and 96% for amphibians.

Synthetic culture water should be prepared from distilled, double deionized water, having a conductivity of 0.25 µmhos or less. Routine monitoring should be conducted for background contaminants. Calcium, magnesium, sodium and potassium salts should be added as given in Table 3. This basic stock is prepared to give a water hardness level of 200 ppm $CaCO_3$ and a pH of 7.5-8.0. Different levels of hardness may be achieved by dilution of the basic stock water, according to directions given in Appendix II. Should regulation of pH prove necessary, adjustments may be made by altering the concentration of sodium bicarbonate and/or by addition of dilute sulfuric acid or sodium hydroxide (55, 63).

This basic stock solution (Table 3) has essentially the same pH, conductivity and water hardness as given for Mount's test water, which consisted of a mixture of natural spring water (emerging from limestone strata) and carbon-filtered, deionized tap water (55). Also, the content of calcium, magnesium and potassium is in close agreement. The concentrations of cations and anions listed in Table 3 fall within ranges published for freshwater resources in Arizona (56), Kentucky (57), and other areas of the U.S. (58). Total chloride content, total dissolved solids, and the concentration of sodium plus potassium are well under maximum levels of 170 mg/l, 400 mg/l and 85 mg/l, observed in 95% of U.S. waters found to support good, mixed fish fauna (59). Also, specific conductivity of 300 µmhos falls within the range of 150-500 recommended for fish propagation (58), and is typical of values recorded for freshwater resources in Kentucky (57). In addition, osmolarity is well within the maximum limit of 50 mOsm/Kg water suggested for freshwaters in the U.S. (47). Total alkalinity exceeds the minimum recommended level of 20 mg/l $CaCO_3$, and closely approaches the range cited as optimum to support a diversified aquatic life (47, 58). As maintained in the culture system

TABLE 3

SYNTHETIC CULTURE WATER

DISSOLVED SALTS[1] (mg/l)	
$CaCl_2$	150
$MgSO_4 \cdot 7H_2O$	150
$NaHCO_3$	100
KCl	5
CHEMICAL COMPOSITION (mg/l)	
Ca	54.2
Mg	14.8
Na	27.4
K	2.6
Cl	98.2
HCO_3	72.6
SO_4	58.5
Total dissolved solids	328.3
PHYSICOCHEMICAL CHARACTERISTICS[2]	
Hardness (as mg/l $CaCO_3$)	200.0
Total alkalinity (as mg/l $CaCO_3$)	82.0
Conductivity (μmhos/cm)	300.0
Osmolarity (mOsm/Kg H_2O)	12.0
pH	7.9

[1]Prepared in distilled, deionized water (specific conductivity of 0.2 μmhos).

[2]Measurements made at 22° C. Above figures represent mean values for six measurements.

described herein (continuous aeration), the dissolved oxygen level is approximately 10 mg/l at the culture temperature used for trout embryos (13° C). A minimum of 7 mg/l has been recommended for trout and salmon spawning waters (47). The pH of our culture medium also is well within the range found in natural waters. Bass Becking et al. (60) observed a pH range of 6-8 for 85% of 347 freshwater rivers and lakes surveyed, and pH values of 6.5-8.5 generally are found in the more productive freshwater resources in the U.S. (47).

Test conditions and monitoring procedures. In the initial characterization of an environmental toxicant, water quality parameters discussed above (Table 3) should be held constant, while the concentration of the toxicant is varied to determine dose/response data. Using the bioassay system as recommended, the common physicochemical parameters of the test water should fall within ranges prevailing for most U.S. freshwaters, facilitating extrapolations from test data. After initial dose/response studies are completed for a selected toxicant, certain water quality variables (e.g., pH, hardness, temperature) may be manipulated independently to determine whether they affect test responses exerted by the toxicant.

In initial investigations, if only one water hardness level is to be selected, we recommend using 100 or 200 ppm $CaCO_3$, with a pH of 7.5-8.0 (Table 3; Appendix II). Culture water should be given continuous aeration (filtered air) supplied either to the mixing chamber or to the reservoir serving the peristaltic pump (Figure 2). The latter is advised when more volatile toxicants are used. Temperature should be maintained within the range considered optimum for development of the selected animal test species, preferably holding overall fluctuations to 1° C. It should be noted that the rate of embryonic development for fish and amphibians will vary with temperature. Significant increases in temperature will shorten hatching time, providing the tolerance limit of the species is not exceeded. Concerning test species which we have used most frequently in the bioassay system described above, average hatching times with preferred temperatures are 24 days for rainbow trout (13° C), 6 days for channel catfish (22° C), 5 days for goldfish (19° C), 4 days for black bass (18° C) and 6-3.5 days for the leopard frog (18-25° C). Data for additional species are given in Table 10.

Bioassay cultures preferably should be maintained in walk-in, temperature regulated environmental rooms. All cultures must be monitored once or twice

daily for temperature, dissolved oxygen, ammonia, water hardness and pH. For these measurements we use a YSI tele-thermometer with thermocouple, YSI oxygen meter (model 51A), Orion ammonia and water hardness electrodes, and a Corning digital pH meter (model 110, with expanded millivolt scale). The accuracy with which general water parameters may be regulated within this test system is illustrated in Table 4, which summarizes results for a series of 18 bioassays conducted with boron compounds at water hardness levels of 50 and 200 ppm $CaCO_3$ and pH levels varying from 7.5-8.5.

Particular attention must be given to trout embryos, especially during the "green stage." Harmful exposure to artificial light should be precluded (61, 62) and it is advisable to maintain cultures under semi-sterile conditions to minimize occurrences of soft egg disease or fungus. Prior to each use, culture rooms are disinfected and irradiated with UV for 12 hours, and culture dishes and other glassware are chemically cleansed and autoclaved. Culture rooms may be maintained under positive pressure to minimize contamination. In the event of fungus or soft egg disease, periodic treatment may be given with formaldehyde (1/1000 for 10 minutes), acriflavine (1/2000 for 20 minutes), or other procedures given in Appendix II.

Flow rates from peristaltic and syringe pumps must be monitored at least twice daily, and the toxicant exposure level should be analyzed at regular, 12-24-hr intervals, as discussed below (Monitoring of Toxicant). Test organisms must be examined daily to gauge extent and frequency of development, and to remove dead specimens. Control eggs should be cultured simultaneously with experimentals and under identical conditions, except for the omission of the selected toxicant.

In bioassays with metallic toxicants, we generally have maintained flow rates at 200, 300 and 600 ml/hr, giving turnover times for 300 ml cultures of 1.5, 1.0 and 0.5 hours, respectively. As seen in Table 11, we have not found flow rate to constitute a substantial test variable, providing it is sufficiently high to prevent deterioration of culture water. For water soluble toxicants of low volatility, we recommend a flow rate which will provide a culture turnover time of 0.5-1.0 hour. However, flow rate requirements may differ significantly for certain organic toxicants. Accordingly, some experimentation may be required to determine effects of flow rate on selected organics. In particular, higher flow rates may be required to prevent hydrophobic organics from partitioning in the culture chambers.

Monitoring of toxicant. It is essential that actual exposure levels be

determined by direct chemical analysis of culture water. Each culture, including controls, should be analyzed daily, using duplicate or triplicate samples, and mean exposure ± standard error should be determined for the bioassay treatment period. The continuous flow test system should be operated 24-72 hours prior to the introduction of test organisms. During this period, toxicant exposure levels and other test parameters should be stabilized.

Particular care should be given to toxicant levels during the first 24 hours following initiation of the egg cultures. When toxicants of high bioaccumulation potential are administered at ppb levels, the exposure concentration may drop sharply when eggs are first added to the test system, and adjustments in the toxicant injection rate may be required.

The method of chemical analysis to be used for culture monitoring should be chosen carefully, considering detection limits, reproducibility, sample volume requirements, and operational efficiency. Simultaneous operation of 20-30 bioassay units will impose a considerable burden on analytical facilities. However, culture assays are essential on a daily basis, to confirm exposure levels and to provide necessary data for adjustments in the test systems. Generally, exposure levels can be held to an overall variation of 5-10%. Proficiency in analytical techniques should be achieved before bioassay tests are undertaken, and alternative procedures should be used to confirm the reliability of the chosen monitoring method.

Considering the vast array of environmental toxicants which may require screening, the bioassay technician may have recourse to a wide variety of analytical procedures. Consultation with analytical chemists may help insure proper choice and execution of detection methods. Two forms of instrumentation which we have used extensively in bioassay studies include atomic absorption spectrophotometry and gas chromatography. The former provides reliable detection for a wide range of elements which may appear as trace contaminants in water used for toxicological studies. In a present investigation on coal toxicology, a Perkin-Elmer model 503 AAS unit, equipped for digital readout and data recording, is being used to monitor inorganic trace elements. When operated with a cold-vapor kit or graphite furnace, low detection limits are achievable for mercury, cadmium and many other elements (Table 12).

In the bioassay evaluation of a series of organic toxicants, we are

using gas chromatography (Packard 7400 with data integrator) as the principal monitoring technique. Table 13 gives detection limits and recommended GC procedures for several classes of organics.

Backup analyses for organic compounds are performed by GC-mass spectroscopy (Finnigan model 3300 F). The latter is particularly useful for monitoring control and experimental test water for background contaminants.

Test concentrations and exposure time. Depending upon the degree of anticipated toxicity, solubility and analytical detection limits, bioassay tests generally are initiated at 10-100 parts per million (10-100 mg/l) toxicant and continued at 10-fold dilutions down to 0.1-1.0 part per billion (0.1-1.0 µg/l). If necessary, this range of test concentrations may be extended to provide adequate delineation of threshold and LC_{50} values, or to reflect levels of toxicants reported for natural water resources. Usually, 5 to 6 test concentrations are sufficient for the initial characterization of a selected toxicant. It is often desirable to repeat the exposure series for two or more water hardness levels (e.g., 50 and 200 ppm $CaCO_3$).

Generally, toxicants should be administered continuously from fertilization through 4-7 days posthatching. At 4 days posthatching, using recommended temperatures, exposure periods will approximate 28, 10, 9 and 8 days for trout, catfish, goldfish and bass, respectively. In bioassays with fish eggs, it appears that certain environmental contaminants (e.g., Hg, B) are more toxic to early embryos, while other pollutants (e.g., Cu, Zn, PCB) are more lethal to late embryonic stages and/or early fry (3, 5, 24-30, 45). Exposure times noted above cover the full range of embryonic events, as well as hatching and the sensitive early juvenile period, and are advisable to adequately evaluate the effects of environmental toxicants on the development and growth of immature piscine stages. However, shorter exposure periods may be used for initial rapid-screening procedures.

Sample size and test responses. Control eggs should be cultured simultaneously with experimentals and under identical conditions, except for the omission of the selected toxicant. With proper daily maintenance, viable, fertilized eggs should give hatching frequencies in control populations of 85% or more (e.g., Table 14). In the event control survival drops below 75%, attention should be given to the source of eggs and sperm, as well as to culture conditions (Appendix II).

A sample size of 100-150 eggs (2-3 replicates) is recommended for both

experimental and control cultures. Quantification of test responses generally should be limited primarily to lethality, teratogenesis and locomotor impairment, expressed in each case as frequency among experimental populations/frequency in controls. Dose/response data should be plotted for continuous exposure from fertilization through hatching and 4 days posthatching (e.g., Figures 4, 5; Table 8). Threshold and LC_{50} values should be expressed for individual and combined test responses for embryonic and postembryonic stages, as well as for the entire exposure period from fertilization to 4-7 days posthatching (e.g., Tables 8, 9). Log-probit analysis or other statistical procedures should be used to determine significance of test responses (e.g., Table 9).

Observations on teratogenesis may be restricted to debilitating anomalies affecting those animals which survive the hatching process. Examples of normal and teratogenic trout alevins are given in Plates I-VI of Appendix III. In the analysis of teratogenic defects, particular attention should be given to the skeletal system, especially the vertebral column. The latter appears to be the single most reliable indicator of teratogenic action in fish. This is apparent in Table 15, where we have summarized frequencies of boron-induced anomalies for several aquatic species. As seen in Plates II-IV, development of the vertebral column, probably sclerotomial differentiation, also is affected by a number of metallic toxicants (e.g., Cd, Hg). Rainbow trout and the channel catfish are particularly suitable test species for screening teratogenic compounds. As seen in Table 16, trout embryos exhibit especially high frequencies of teratogenesis for a wide range of metallic toxicants.

Selection of animal test species. Over the past 4 years we have worked with approximately 20 fish and amphibian species. Most are adaptable for use with the bioassay system described above. The fish which we have used most extensively include the black bass (Micropterus salmoides), goldfish (Carassius auratus), channel catfish (Ictalurus punctatus), and the rainbow trout (Salmo gairdneri). Amphibian species, all of which are Anurans, include the red-spotted toad (Bufo punctatus), southern gray treefrog (Hyla chrysocephala), narrow-mouthed toad (Gastrophryne carolinensis), squirrel treefrog (Hyla squirella), pig frog (Rana grylio) and the leopard frog (Rana pipiens). Hatching times, egg production, seasonal availability and possible sources of supply are summarized in Table 10. Numerous other species also are suitable for test purposes, including the fathead minnow (Pimephales promelas), bluntnose minnow (Pimephales notatus), bluegill

(Lepomis macrochirus) and the zebra fish (Brachydanio rerio).

In selecting test species, consideration should be given to availability, ease of handling in the laboratory, geographical and ecological distribution, pattern of reproduction and development, and sensitivity to toxicants. Sensitivity of eggs and embryos may vary with hatching time, egg yolk volume, and certain other developmental features. For example, in rapid-scan bioassays in which culture water and toxicant were renewed at 12-hr intervals, we observed up to 100-fold variations in mercury tolerances of various fish and amphibian eggs (Figure 6; Tables 17, 18). Generally, mercury sensitivity of fish stages increased with hatching time and/or egg yolk volume. However, species sensitivity does not remain constant for all classes of toxicants. Thus, for in-depth bioassay investigations, we recommend selecting 2 to 3 test species which differ in important developmental characteristics. Such tests will provide a more reliable and representative index to the embryopathic effects produced by environmental trace contaminants.

In comparing the sensitivity of developmental stages of different fish and amphibian species to particular toxicants, it is important to regulate exposure period to cover essentially the same embryonic and post-embryonic stages. This is especially important as both the order and degree of sensitivity of different developmental stages may vary substantially for different toxicants. The recommended exposure period largely encompasses all developmental processes from fertilization through yolk sac resorption, providing a basis for comparative toxicological studies. As developmental (exposure) time may vary for different test species, it should be treated appropriately as a significant test variable.

APPENDIX I

SUPPORTING DATA

FIGURES 3-6

TABLES 4-19

FIGURE 3

CONTINUOUS FLOW BIOASSAY SYSTEM

3.1 Series of egg culture dishes (A) which receive toxicant and culture water from mixing chamber (B). Culture water is supplied to mixing chambers by peristaltic pump (C). Liquid flow meters (D) are used to measure flow rate.

3.2 Enlarged view of culture room showing culture dishes (A), mixing chambers (B), peristaltic pumps (C), and flow meters (D). Culture dishes exhaust water into plexiglass drain troughs which are connected to waste reservoirs.

3.3 Syringe pump used to supply toxicant to mixing chamber. Syringe pumps are mounted on outside culture room wall to avoid mechanical problems resulting from culture room temperature and humidity. Note the modified holder which supports six 10 ml syringes. A modified syringe mounting bracket is shown inverted on the syringe drive carriage (arrow).

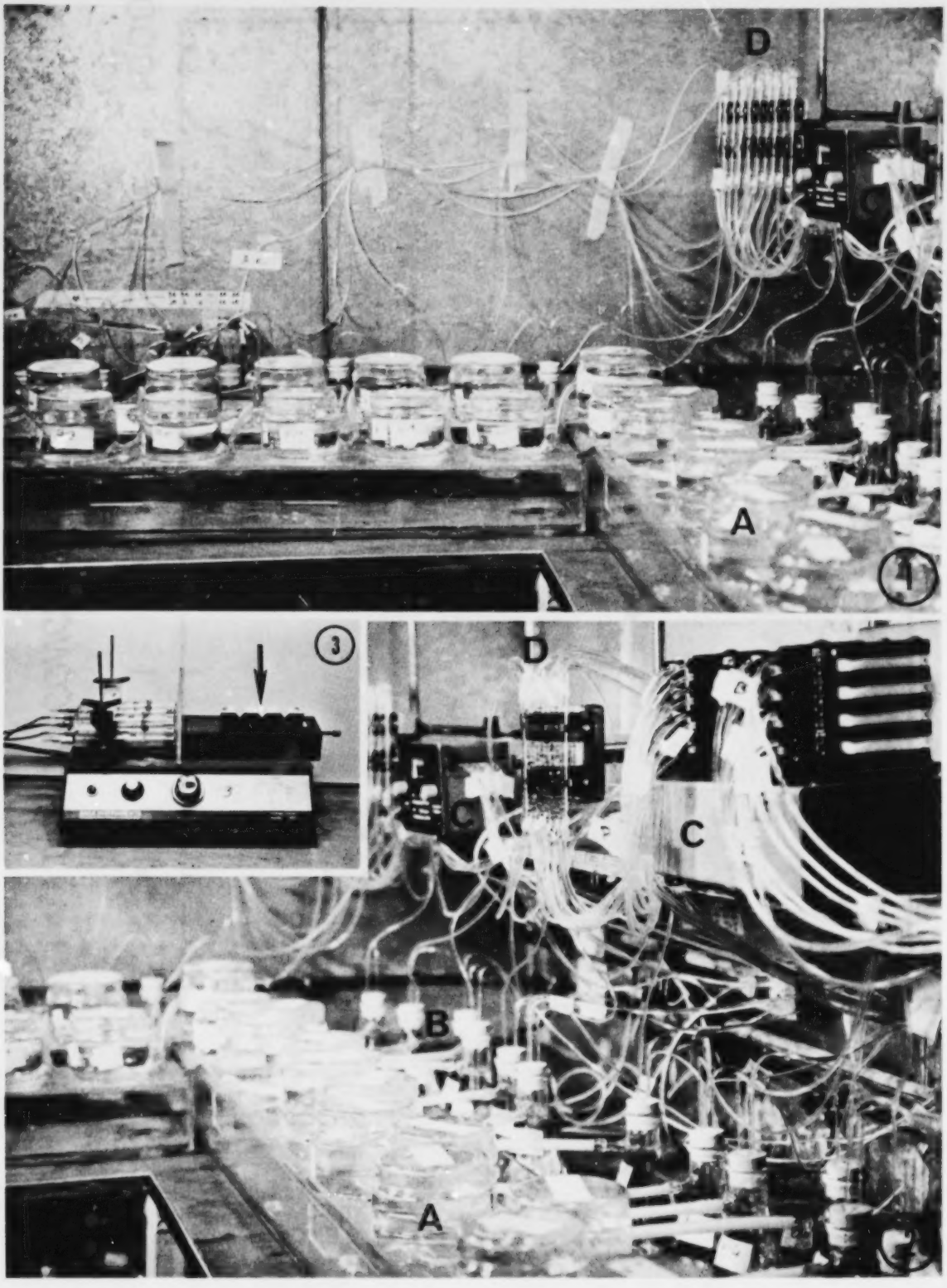
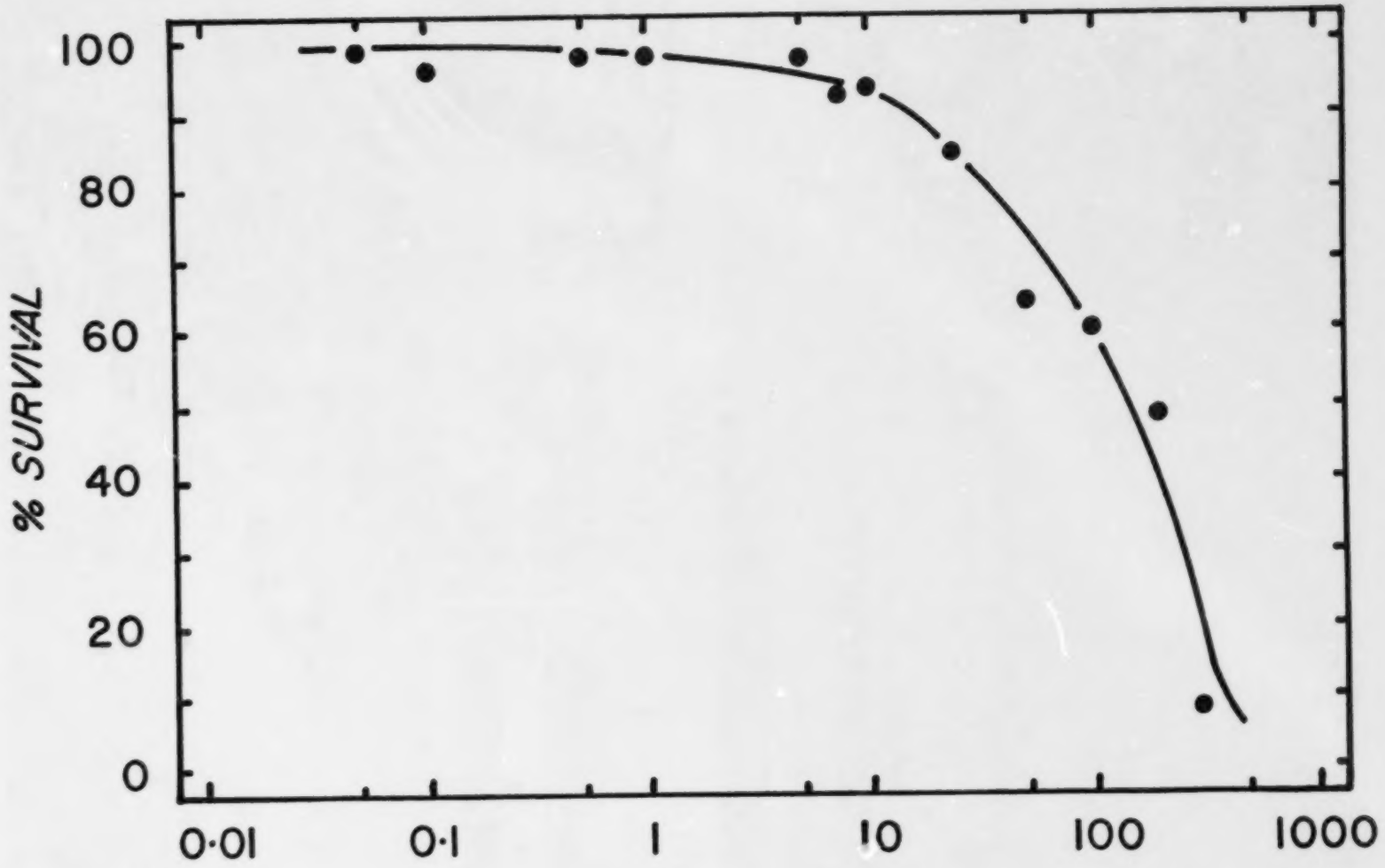
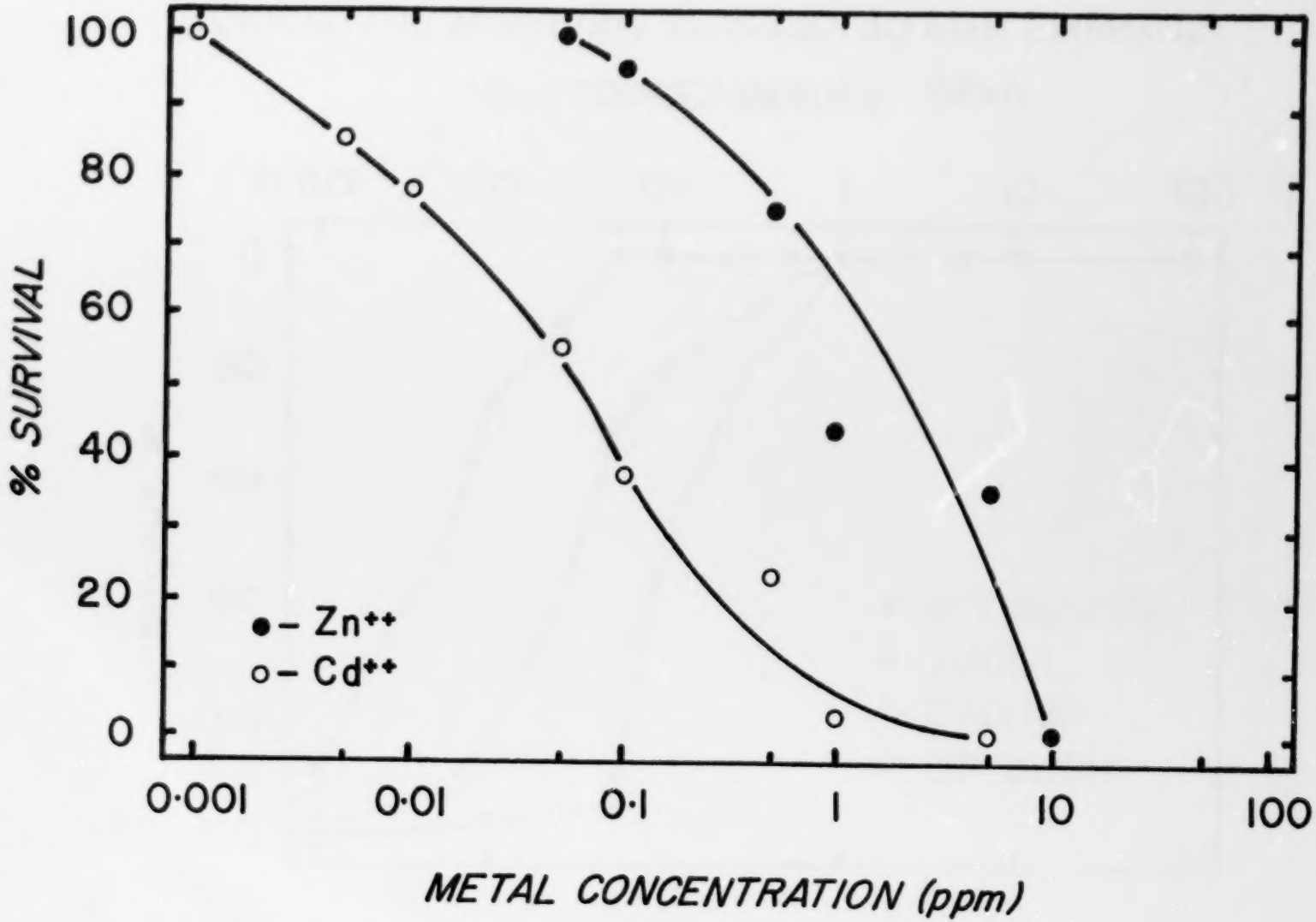

D
A
1
3
D
C
C
B
A

FIGURE 4



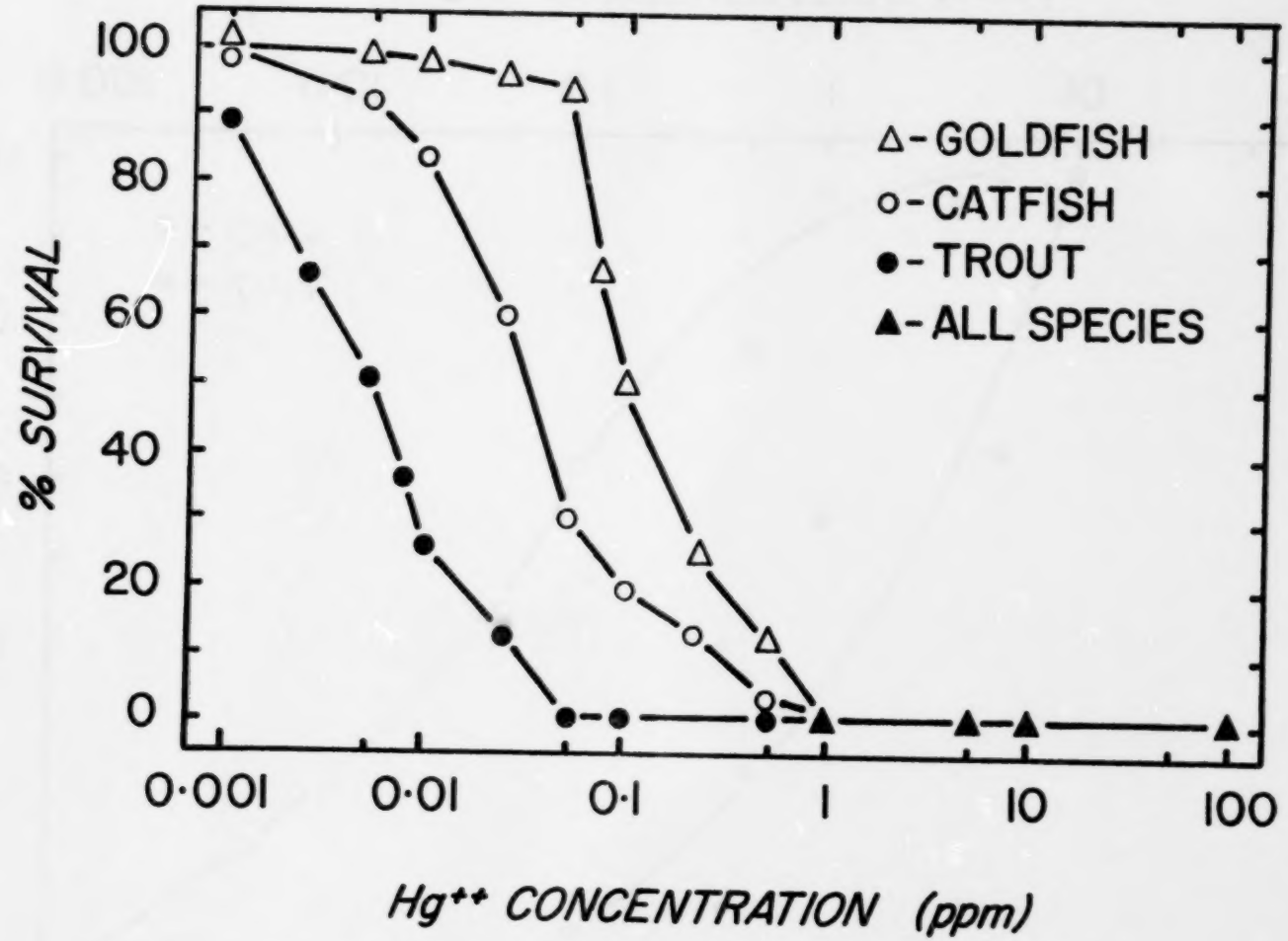
TOXICITY OF BORIC ACID TO GOLDFISH EMBRYOS

FIGURE 5



TOXICITY OF METALS TO GOLDFISH EMBRYOS

FIGURE 6



TOXICITY OF INORGANIC MERCURY TO FISH EMBRYOS

TABLE 4

TEST PARAMETERS OBSERVED DURING BIOASSAYS WITH BORON COMPOUNDS

BIOASSAYS (EMBRYONIC & POSTEMBRYONIC STAGES)		OBSERVED TEST PARAMETERS (MEAN ± STANDARD ERROR)				
TEST SPECIES	COMPOUND/WATER HARDNESS	TEMPERATURE (°C)	DISSOLVED OXYGEN (ppm)	WATER HARDNESS (ppm $CaCO_3$)	pH	FLOW RATE (ml/hr)
CATFISH	BORIC ACID @					
	50 ppm $CaCO_3$	25.0 ± 0.4	7.3 ± 0.1	51.8 ± 2.0	7.5 ± 0.1	194.2 ± 4.3
	200 ppm $CaCO_3$	24.7 ± 0.6	7.6 ± 0.1	212.0 ± 11.0	7.6 ± 0.0	199.7 ± 2.7
	BORAX @					
	50 ppm $CaCO_3$	29.4 ± 0.3	6.4 ± 0.1	46.7 ± 2.3	8.5 ± 0.0	188.8 ± 4.1
	200 ppm $CaCO_3$	29.4 ± 0.3	6.5 ± 0.1	194.7 ± 3.2	8.2 ± 0.1	191.0 ± 1.8
FOWLER'S TOAD	BORIC ACID @					
	50 ppm $CaCO_3$	23.7 ± 0.6	6.8 ± 0.1	57.4 ± 4.2	7.6 ± 0.1	185.3 ± 2.8
	200 ppm $CaCO_3$	23.7 ± 0.6	6.8 ± 0.1	207.5 ± 10.0	7.6 ± 0.1	189.8 ± 4.6
GOLDFISH	BORIC ACID @					
	50 ppm $CaCO_3$	24.8 ± 0.3	7.4 ± 0.0	54.4 ± 4.2	7.9 ± 0.1	184.6 ± 5.7
	200 ppm $CaCO_3$	24.8 ± 0.3	7.5 ± 0.0	207.5 ± 10.0	7.6 ± 0.1	180.4 ± 5.4
	BORAX @					
	50 ppm $CaCO_3$	27.0 ± 0.4	7.5 ± 0.2	46.2 ± 1.8	8.3 ± 0.1	208.0 ± 4.5
	200 ppm $CaCO_3$	27.0 ± 0.4	7.5 ± 0.2	194.8 ± 16.0	8.1 ± 0.1	224.2 ± 3.0
LEOPARD FROG	BORIC ACID @					
	50 ppm $CaCO_3$	25.0 ± 0.3	7.7 ± 0.2	52.5 ± 1.4	7.7 ± 0.0	193.4 ± 2.3
	200 ppm $CaCO_3$	25.0 ± 0.3	7.8 ± 0.2	212.3 ± 5.4	7.7 ± 0.0	189.2 ± 4.4
	BORAX @					
	50 ppm $CaCO_3$	25.3 ± 0.2	7.7 ± 0.2	46.2 ± 4.9	8.3 ± 0.1	180.0 ± 4.1
	200 ppm $CaCO_3$	25.3 ± 0.2	7.8 ± 0.2	203.0 ± 2.3	8.4 ± 0.1	195.6 ± 4.1
TROUT	BORIC ACID @					
	50 ppm $CaCO_3$	13.7 ± 0.1	9.2 ± 0.1	54.1 ± 3.5	7.7 ± 0.1	195.0 ± 1.2
	200 ppm $CaCO_3$	13.3 ± 0.1	9.6 ± 0.1	204.0 ± 4.0	7.9 ± 0.1	189.4 ± 2.1
	BORAX @					
	50 ppm $CaCO_3$	14.0 ± 0.1	10.1 ± 0.2	49.0 ± 1.4	7.9 ± 0.1	190.4 ± 2.9
	200 ppm $CaCO_3$	13.0 ± 0.1	10.3 ± 0.2	191.0 ± 4.0	7.8 ± 0.1	189.4 ± 2.1

TABLE 5

TABLE 5A. ANALYSIS OF FLOW RATES FOR BRINKMAN PERISTALTIC PUMP (MODEL 131900)[1]

Flow Rate (ml/hr)	Channel Number[2] 1	2	3	4	5	6
50	49.8±0.3	49.5±0.2	49.2±0.1	50.0±0.6	50.8±0.2	50.1±0.2
100	99.4±0.4	97.4±0.5	98.6±0.3	101.5±0.4	101.6±0.4	99.7±0.3
150	150.8±2.4	146.6±1.0	150.2±0.8	150.9±0.9	149.1±1.2	151.4±1.2
300	299.9±2.0	294.6±2.7	296.1±2.0	304.6±2.0	302.2±2.1	297.7±2.3

1 Tygon tubing - 1/16" ID, 1/8" OD.

2 Mean ± standard error; each value based on 20 replicates over a 7 day period.

TABLE 5B. ANALYSIS OF FLOW RATES FOR GILSON PERISTALTIC PUMP (MODEL HP8)[1]

Flow Rate (ml/hr)	Channel Number[2] 1	2	3	4	5	6
50	48.7±0.4	50.4±0.7	50.8±0.6	48.7±0.4	48.3±0.0	48.3±0.0
100	96.7±0.4	100.5±0.5	99.9±0.5	100.1±0.5	96.5±0.4	96.5±0.4
175	170.4±0.6	173.6±0.6	177.2±0.5	173.8±0.6	172.4±0.4	169.4±0.5
275	267.3±0.8	273.9±2.0	278.5±0.9	274.1±0.6	268.5±0.7	265.5±0.7

1 Tygon tubing - 1/16" ID, 1/8" OD.

2 Mean ± standard error; each value based on 20 replicates over a 7 day period.

TABLE 5C. ANALYSIS OF DELIVERY RATES FOR SAGE SYRINGE PUMP (MODEL 355)

Flow Rate (ml/hr X 100)	Syringe Number[1] 1	2	3	4	5	6
10.00[2]	10.07±0.24	9.80±0.17	10.00±0.16	10.21±0.21	9.53±0.13	10.42±0.19
30.00[2]	31.40±0.46	31.80±0.48	32.00±0.37	31.50±0.48	31.50±0.50	32.60±0.36
30.00[3]	30.14±0.57	30.09±0.67	32.05±0.76	31.13±0.82	30.50±0.58	30.14±0.61
60.00[3]	64.00±1.24	57.11±2.20	60.38±1.31	63.79±1.24	61.67±2.16	64.00±1.15

1 Mean ± standard error; each value based on 20 replicates over a 7 day period.

2 10 ml glass syringe (single ground).

3 10 ml plastic disposable syringe.

TABLE 6

CORRELATION OF PERCENT SURVIVAL WITH EXPOSURE TIME

FOR TROUT EMBRYOS TREATED WITH INORGANIC MERCURY IN A CONTINUOUS FLOW SYSTEM

CULTURE NUMBER		EXPOSURE TIME IN DAYS 1	2	3	4	5	6	7	8	MEAN EXPOSURE[1]
1	% SURVIVAL	100	99	95	82	59	41	23	0	
	ppb Hg^{+2}	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.11 ± 0.01
2	% SURVIVAL	100	97	94	79	55	39	26	0	
	ppb Hg^{+2}	0.1	0.1	0.2	<0.1	0.1	0.1	0.2	0.3	0.14 ± 0.03
3	% SURVIVAL	100	100	97	83	57	49	26	0	
	ppb Hg^{+2}	0.1	0.1	0.1	<0.1	<0.1	0.1	0.2	0.2	0.10 ± 0.03

[1]Mean exposure ± standard error for the 8 day period.

TABLE 7

EXPOSURE OF RAINBOW TROUT EMBRYOS AND ALEVINS (SALMO GAIRDNERI)

TO CADMIUM IN WATER OF 100 PPM $CaCO_3$ HARDNESS

CONCENTRATION[1] CADMIUM (PPB)	TEST RESPONSES AT HATCHING (H) AND 4 DAYS POSTHATCHING (PH)				
	% HATCHABILITY[2]	% NORMAL SURVIVAL[3]		% DEAD OR TERATOGENIC	
		H	PH	H	PH
5.16 ± 0.44	94(2)	92	92	8	8
11.52 ± 0.82	93(3)	90	89	10	11
27.95 ± 1.70	86(8)	79	67	21	33
50.95 ± 2.51	76(9)	69	63	31	37
79.52 ± 3.97	72(10)	65	62	35	38
103.20 ± 4.09	78(23)	60	55	40	45
254.00 ± 7.00	55(100)	0	0	100	100

[1]Concentration given as mean ± standard error as determined by atomic absorption spectrophotometry.

[2]Percent of surviving population bearing gross teratogenic defects is given parenthetically.

[3]Percent of initial population surviving with normal morphology.

TABLE 8

EXPOSURE OF CATFISH EMBRYOS AND FRY (ICTALURUS PUNCTATUS)

TO BORON (BORIC ACID) IN WATER OF 200 PPM $CaCO_3$ HARDNESS

CONCENTRATION BORON (PPM) NOMINAL	ACTUAL[1]	TEST RESPONSES AT HATCHING (H) AND 4 DAYS POSTHATCHING (PH)				
		% HATCHABILITY[2]	% NORMAL SURVIVAL[3]		% DEAD OR TERATOGENIC	
			H	PH	H	PH
0.01	- -	100(2)	98	98	2	2
0.05	- -	100(1)	99	98	1	2
0.50	0.53 ± 0.02	100(2)	98	94	2	6
0.75	0.77 ± 0.01	100(2)	98	98	2	2
1.00	0.96 ± 0.01	96(3)	93	86	7	14
2.50	2.33 ± 0.12	99(2)	97	87	3	13
5.00	4.90 ± 0.07	95(4)	91	82	9	18
7.50	7.40 ± 0.10	95(4)	91	75	9	25
10.00	9.43 ± 0.23	94(8)	87	65	13	35
25.00	25.10 ± 0.27	90(28)	65	40	35	60
50.00	48.30 ± 0.84	83(30)	58	28	42	72
75.00	77.70 ± 0.54	71(43)	40	9	60	91
150.00	140.00 ± 5.00	57(22)	44	2	56	98
300.00	302.00 ±29.00	0	0	0	100	100

[1]Concentration given as mean ± standard error as determined by curcumin method.

[2]Percent of surviving population bearing gross teratogenic defects is given parenthetically.

[3]Percent of initial population surviving with normal morphology.

TABLE 9

LOG PROBIT ANALYSIS OF THRESHOLD (LC_1) VALUES IN PPM

FOR AQUATIC EMBRYOS EXPOSED TO BORON COMPOUNDS[1]

TEST SPECIES	BORIC ACID				BORAX			
	50 ppm $CaCO_3$		200 ppm $CaCO_3$		50 ppm $CaCO_3$		200 ppm $CaCO_3$	
	H	PH	H	PH	H	PH	H	PH
CATFISH	1.0	0.5	0.3	0.2	5.5	5.5	1.7	1.7
FOWLER'S TOAD	25.0	25.0	5.0	5.0	-	-	-	-
GOLDFISH	0.6	0.6	0.2	0.2	1.4	1.4	0.9	0.9
LEOPARD FROG	26.0	13.0	23.0	22.0	6.0	5.0	3.0	3.0
TROUT	0.1	0.1	0.001	0.001	0.07	0.07	0.07	0.07

[1]Threshold values were calculated at hatching (H) and 4 days posthatching (PH) for two water hardness levels (50 ppm $CaCO_3$, 200 ppm $CaCO_3$). Grossly anomalous survivors were counted as lethals.

TABLE 10

SPECIES SELECTED FOR USE AS EMBRYONIC BIOINDICATORS

SPECIES	Developmental Time in Days	Number of Eggs* Per Female	Breeder's Availability	Source
Largemouth Bass (Micropterus salmoides)	4 (18°C)	10,000-15,000	April-June	National Fish Hatchery Frankfort, Kentucky
Goldfish (Carassius auratus)	5 (19°C)	15,000-25,000	March-July	National Fish Hatchery Frankfort, Kentucky
Channel Catfish (Ictalurus punctatus)	6 (22°C)	10,000-15,000	May-August	National Fish Hatcheries Frankfort, Ky. and Senacaville, Ohio
Rainbow Trout (Salmo gairdneri)	24 (13°C)	6,000-8,000	Sept.-April	National Fish Hatchery Wytheville, Virginia
Pig Frog (Rana grylio)	6 (22°C)	6,000-10,000	March-June	Charles D. Sullivan Nashville, Tennessee
Leopard Frog (Rana pipiens)	6 (19°C)	2,000-4,000	Dec.-May	Amphibian Facility, Univ. Michigan, Ann Arbor
Red-spotted Toad (Bufo punctatus)	3 (22°C)	2,000-4,000	April-August	Charles D. Sullivan Nashville, Tennessee
Narrow-mouthed Toad (Gastrophryne carolinensis)	3 (22°C)	1,000-3,000	May-August	Charles D. Sullivan Nashville, Tennessee
Southern Gray Treefrog (Hyla chrysocephala)	3 (22°C)	100-500	May-June	Charles D. Sullivan Nashville, Tennessee
Squirrel Treefrog (Hyla squirella)	3 (22°C)	80-150	May-August	Charles D. Sullivan Nashville, Tennessee

*Egg production dependent upon size and maturity.

TABLE 11

EFFECT OF FLOW RATE ON

METAL TOXICITY TO CATFISH EMBRYOS AND ALEVINS

FLOW RATE (ml/hr)	TURNOVER RATE (per hour)[1]	PERCENT SURVIVAL AT HATCHING (H) AND 4 DAYS POSTHATCHING (PH) FOR METAL CONCENTRATIONS IN PPM								
		Hg^{+2}	H	PH	Cd^{+2}	H	PH	Zn^{+2}	H	PH
50	0.2	0.0021	36	17	0.23	40	13	0.42	57	40
125	0.5	0.0027	45	23	0.25	48	22	0.50	59	38
250	1.0	0.0025	46	25	0.29	54	32	0.50	60	34
400	1.6	0.0024	43	25	0.30	50	27	0.59	61	35

[1]Values represent fraction of a 250 ml culture chamber renewed per hour.

TABLE 12

COAL TRACE ELEMENTS SELECTED FOR BIOASSAY EVALUATIONS

Trace Element		Concentration[1] in Coal (ppm)	Bioassay Test Compound	Detection Limit (AAS)
Aluminum	(Al)	10,440-12,900	$AlCl_3$	0.20 ppb
Antimony	(Sb)	0.50-1.26	$SbCl_3$	2.00 ppb
Arsenic	(As)	4.45-14.02	$NaAsO_2$	0.80 ppb
Cadmium	(Cd)	0.47-2.52	$CdCl_2$	0.01 ppb
Cobalt	(Co)	2.90-9.57	$CoCl_3$	1.60 ppb
Chromium	(Cr)	13.75-18.00	$CrCl_2$	0.40 ppb
Copper	(Cu)	8.30-15.16	$CuCl_2$	0.20 ppb
Germanium	(Ge)	1.00-6.59	GeO_2	0.20 ppm
Lanthanum	(La)	3.80-10.00	$LaCl_3$	2.00 ppm
Lead	(Pb)	4.90-34.78	$PbCl_2$	0.20 ppb
Manganese	(Mn)	33.80-49.40	$MnCl_2$	0.04 ppb
Mercury	(Hg)	0.12-0.20	$HgCl_2$	0.10 ppb
Molybdenum	(Mo)	5.00-7.54	MoO_2Cl_2	2.00 ppb
Nickel	(Ni)	16.00-21.07	$NiCl_2$	2.00 ppb
Silver	(Ag)	0.03-0.12	$AgNO_3$	0.02 ppb
Selenium	(Se)	2.08-2.20	Na_2SeO_4	2.00 ppb
Strontium	(Sr)	10.00-23.00	$SrCl_2$	1.00 ppb
Thallium	(Tl)	0.29-2.00	$TlCl_3$	0.40 ppb
Tin	(Sn)	0.03-4.79	$SnCl_2$	4.00 ppb
Tungsten	(W)	0.10-3.00	WO_2Cl_2	1.00 ppm
Vanadium	(V)	28.50-32.71	V_2O_5	0.02 ppm
Zinc	(Zn)	46-272	$ZnCl_2$	0.004 ppb

[1]The majority of values are from Ruch et al. (9), Fulkerson et al. (10), and Carter (11), and represent means for multiple coal samples taken largely from Western Kentucky and Southern Illinois. Lower means for Ag, Tl, Sn and W are from Lloyd (39), and the upper mean for Ag is from Vaughan et al. (4).

TABLE 13. PHYSICAL-CHEMICAL CHARACTERISTICS AND ANALYTICAL PROCEDURES FOR SELECTED ORGANIC CHEMICALS

ORGANIC COMPOUND	CLASS OF COMPOUND	SOLUBILITY mg/l	VAPOR PRESSURE mm Hg @ (°C)	ANALYTICAL PROCEDURES		DETECTION LIMIT ug/l	REFERENCES[2]
				EXTRACTION SOLVENT	DETECTION METHOD[1]		
Aniline	aromatic amine	35000	1.0 (34)	acetone, benzene	A	0.01-.1	1,2,3,5
Atrazine	chlorinated hydrocarbon	33	$3x10^{-7}$ (20)	methylene chloride, hexane	B,C	0.01-.1	4,6,9,12,14,15
Benzidine	aromatic amine	400	1.0 (126)	chloroform, hexane, acetone	D	0.01-.1	2,7,8
Chlorobenzene	chlorinated hydrocarbon	insol.	10.0 (22)	methylene chloride, hexane	B	0.01-.1	1,6,7,9,14,15
Chloroform	chlorinated hydrocarbon	7.2	100.0 (10.4)	cyclohexane, ether, hexane	E,F	0.01-.1	2,7,10
2,4-D	chlorinated hydrocarbon	620	0.4 (160)	methylene chloride, hexane	B	0.01-.1	4,6,9,14,15
Dieldrin	chlorinated hydrocarbon	pract. insol.	$3x10^{-6}$ (20)	chloroform, hexane, acetone	B	0.01-.1	3,4,6,9,12,14,15
Di-2-ethylhexyl-phthalate	carboxylic acid	insol.	1.3 (200)	methylene chloride, hexane	B	0.01-.1	6,7,13
Endrin	chlorinated hydrocarbon	0.23	$2x10^{-7}$ (25)	methylene chloride, hexane, acetone	B	0.01-.1	3,4,6,9,11,12,14
Hexachloro-benzene	chlorinated hydrocarbon	pract. insol.	$1x10^{-5}$ (20)	methylene chloride, hexane	B	0.01-.1	4,6,9,12,14,15
Malathion	organic phosphate	144	$4x10^{-5}$ (30)	ethyl acetate, hexane	G	0.01-.1	4,9,12,14,15
Phenol	aromatic hydrocarbon	67000	1.0 (40)	chloroform	H	2	1,9
Toxaphene	chlorinated hydrocarbon	pract. insol.	0.2-0.4 (25)	methylene chloride, hexane, acetone	B	0.01-.1	4,6,9,12,14,15

[1,2]See following page.

TABLE 13 CONTINUED

[1]Key to Organic Detection Methods

A. GC Flame ionization detector
   Column - 1% SE-30
B. GC Electron capture detector
   Column - 10% DC-200 Anakrom ABS
C. GC Flame ionization detector
   Carbowax 20M on Gas-chromosorb Q
D. GC Alkali flame ionization detector
   Column - 3% OV-17
E. GC Electron capture detector
   Column - 20% Carbowax, 20M with microcoulometric detector
F. GC Electron capture detector
   Column - 3% OV-17
G. GC Electron capture detector
   Column - 4% SE-30 and 6% QF-1
H. Colorimetric-spectrophotometric technique- 4-aminoantipyrine reaction

[2]References to Methods of Organic Analysis

1. The Merck Index, 8th Edition. 1968. (P.G. Stecher, ed., M. Windholz, D.S. Leahy, assoc. eds.), Merck and Company, Inc., Rahway, N.J. p. 84.
2. Handbook of Chemistry and Physics, 55th Edition. 1974-1975. (R.C. Weast, ed.), Chemical Rubber Company, Cleveland, Ohio.
3. Chesters, G., H.B. Pionke, and T.C. Daniel. 1974. In Pesticides in Soil and Water. University of Wisconsin, Madison, Wisconsin. pp. 451-550.
4. Martin, Hubert and Charles R. Worthing. 1974. Pesticide Manual, 4th Edition. British Crop Protection Council, Boots Company Ltd., Nottingham, Eng. 565pp.
5. Fales, Henry M. and John J. Pisano. 1964. In Biomedical Applications of Gas Chromatography. (H.A. Szymanski, ed.), Plenum Press, N.Y. pp.31-87.
6. Hurley, John T. 1974. American Water Works Association Journal, 66: 27-31
7. Condensed Chemical Dictionary, 8th Edition. 1971. (G.C. Hawley, ed.), Van Nostrand Reinhold Company, N.Y. 971 pp.
8. Bruser, D. 1976. Department of Chemistry, University of Kentucky, Personal Communication.
9. Standard Methods for the Examination of Water and Wastewater, 13th Edition. 1971. APHA, AWWA, WPCF, Washington, D.C. pp. 100-107.
10. Cuopra, N.M. and L.R. Sherman. 1972. Anal. Chem. 44(6): 1036-1038.
11. Veith, G.D. and V.M. Comstock. 1975. J. Fish. Res. Bd. Canada 32(10): 1849-1851.
12. Official Methods of Analysis of the Association of Official Analytical Chemists, 12th Edition. 1975. (W. Horwitz, ed.), Association of Official Analytical Chemists, Washington, D.C. 1094 pp.
13. Bryant, H. 1976. Department of Entomology, University of Kentucky, Personal Communication.
14. Pesticide Analytical Manual, Vol. 1. 1975. (B.M. McMahon and L.D. Sawyer, eds.), U.S. Department of Health, Education, and Welfare. Food and Drug Administration, Rockville, Md.
15. Pesticide Analytical Manual, Vol. 2. 1974. (J.R. Markus and B. Pumz, eds.), U.S. Department of Health, Education, and Welfare. Food and Drug Administration, Rockville, Md.

TABLE 14

SURVIVAL FREQUENCIES FOR CONTROL POPULATIONS OF AQUATIC EMBRYOS

EMBRYONIC BIOASSAYS		COMBINED POPULATION SIZE	% CONTROL SURVIVAL[1]	
TEST SPECIES	COMPOUND/WATER HARDNESS		AT HATCHING	AT 4 DAYS POSTHATCHING
CATFISH	BORIC ACID @			
	50 ppm $CaCO_3$	304	93	91
	200 ppm $CaCO_3$	258	94	93
	BORAX @			
	50 ppm $CaCO_3$	371	97	97
	200 ppm $CaCO_3$	368	98	98
FOWLER'S TOAD	BORIC ACID @			
	50 ppm $CaCO_3$	443	92	92
	200 ppm $CaCO_3$	410	94	94
GOLDFISH	BORIC ACID @			
	50 ppm $CaCO_3$	294	94	94
	200 ppm $CaCO_3$	294	93	93
	BORAX @			
	50 ppm $CaCO_3$	249	93	92
	200 ppm $CaCO_3$	249	93	92
LEOPARD FROG	BORIC ACID @			
	50 ppm $CaCO_3$	268	90	89
	200 ppm $CaCO_3$	233	89	87
	BORAX @			
	50 ppm $CaCO_3$	268	90	89
	200 ppm $CaCO_3$	233	89	87
TROUT	BORIC ACID @			
	50 ppm $CaCO_3$	242	91	91
	200 ppm $CaCO_3$	242	91	91
	BORAX @			
	50 ppm $CaCO_3$	252	92	87
	200 ppm $CaCO_3$	252	92	87

[1]Where tests at the two water hardness levels (50 and 200 ppm $CaCO_3$) were conducted simultaneously, experimental results were gauged against the same populations of control animals.

TABLE 15

TERATOGENESIS IN AQUATIC EMBRYOS SURVIVING TREATMENT WITH BORIC ACID AND BORAX

BIOASSAYS			PERCENT DISTRIBUTION OF ANOMALIES BY AREAS AFFECTED					
SPECIES	TOXICANT & HARDNESS	NO. ANIMALS ANOMALOUS[1]	DWARFED BODY	CRANIUM	VERTEBRAL COLUMN	FINS	NERVOUS SYSTEM	YOLK SAC & ABDOMEN
CATFISH	BORIC ACID @							
	50 ppm $CaCO_3$	77	6	3	53	38	-	-
	200 ppm $CaCO_3$	125	2	5	93	-	-	-
	BORAX @							
	50 ppm $CaCO_3$	84	38	2	55	-	-	5
	200 ppm $CaCO_3$	107	21	2	75	-	2	-
GOLDFISH	BORIC ACID @							
	50 ppm $CaCO_3$	65	-	2	93	5	-	-
	200 ppm $CaCO_3$	26	-	-	100	-	-	-
	BORAX @							
	50 ppm $CaCO_3$	44	2	-	93	-	-	5
	200 ppm $CaCO_3$	68	-	-	91	2	-	7
TROUT	BORIC ACID @							
	50 ppm $CaCO_3$	58	2	10	84	-	4	-
	200 ppm $CaCO_3$	278	3	8	80	2	2	5
	BORAX @							
	50 ppm $CaCO_3$	40	10	25	40	3	5	17
	200 ppm $CaCO_3$	33	3	13	63	6	15	-
FOWLER'S TOAD	BORIC ACID @							
	50 ppm $CaCO_3$	810	-	1	16	-	-	83
	200 ppm $CaCO_3$	544	-	1	12	-	-	87
LEOPARD FROG	BORIC ACID @							
	50 ppm $CaCO_3$	209	-	-	6	-	-	94
	200 ppm $CaCO_3$	190	-	-	33	-	-	67
	BORAX @							
	50 ppm $CaCO_3$	155	-	1	49	-	-	50
	200 ppm $CaCO_3$	151	-	1	92	1	-	6

[1]Includes anomalous survivors for all exposure levels

TABLE 16

TERATOGENIC EFFECTS OF METALS ON TROUT EMBRYOS

CONCENTRATION[2] in ppm	PERCENT SURVIVAL AT HATCHING[1]						
	CH_3Hg^+	Hg^{+2}	Cd^{+2}	Cu^{+2}	As^{+3}	Zn^{+2}*	Se^{+6}
0.001	90(3)	90(2)	98(2)	97(4)	-	99	99(2)*
0.002	75(9)	72(8)	94(2)	-	-	-	-
0.005	57(10)	59(12)	97(7)	90(10)	-	100(1)	99(1)*
0.007	46(17)	46(19)	97(4)	-	-	-	-
0.010	33(20)	34(22)	97(6)	92(7)	100	98(1)	90(2)*
0.025	29(35)	24(43)	96(7)	82(8)	100(2)	-	-
0.050	0	0	91(9)	65(11)	99(1)	98(3)	91(1)*
0.075	-	-	82(6)	65(5)	98	98(3)	-
0.100	0	0	58(11)	63(4)	96(5)	99(7)	90(2)*
0.250	-	-	38(32)	45(9)	83(11)	-	-
0.500	0	0	32(28)	24(31)	67(8)	91(2)	85(4)
0.750	-	-	28(26)	0	47(18)	-	-
1.000	-	-	5	0	30(27)	56(4)	82(7)
2.500	-	-	0	0	2	25(8)	75(6)
5.000	-	-	0	-	1	1(100)	52(11)
7.500	-	-	-	-	0	0	50(10)
10.000	-	-	0	-	0	-	41(15)
TL_{50}	0.005	0.005	0.10	0.10-0.25	0.50-0.75	1.00	5.00

[1]Percent survival includes all embryos which completed hatching. Frequencies of survivors exhibiting gross anomalies are given parenthetically.

[2]Concentrations based on content of metal added to Holtfreter's culture water. Asterisks designate cultures where NaCl content was reduced to 0.1 g/l.

TABLE 17

TOXICITY OF INORGANIC MERCURY ($HgCl_2$) TO FISH EMBRYOS

CONCENTRATION (ppm)	PERCENT SURVIVAL AT HATCHING[1]			
	TROUT(24)	CATFISH(6)	GOLDFISH(3)	BASS(4)
0.001	89	100	100	99
0.002	66	97	100	96
0.005	51	92	99	95
0.007	37	91	99	92
0.010	26	84	98	86
0.025	13	60	96	83
0.050	0	30	94	81
0.075	-	26	66	70
0.100	0	20	50	68
0.250	-	14	25	39
0.500	0	1	13	14
1.000	0	0	0	0
5.000	0	0	0	0
10.000	0	0	0	0
TL_{50}	0.005	0.025	0.10	0.10-0.25

[1]Hatching time in days is given parenthetically.

TABLE 18

TOXICITY OF INORGANIC MERCURY TO AMPHIBIAN EMBRYOS

CONCENTRATION (ppm)	PERCENT SURVIVAL AT HATCHING							
	Gastrophryne carolinensis[3]	*Hyla squirella*[3]	*Hyla chryso-cephala*[3]	*Rana pipiens*[6]	*Bufo fowleri*[3]	*Bufo punctatus*[3]	*Rana grylio*[6]	*Rana heckscheri*[6]
0.001	51	80	82	96	99	100	100	100
0.002	35	-	64	87	100	100	-	-
0.005	22	44	44	72	100	100	99	100
0.007	0	-	13	62	100	99	96	100
0.010	0	0	0	47	98	92	94	94
0.025	0	-	0	34	79	83	89	90
0.050	0	0	0	9	18	35	75	64
0.075	0	-	0	1	8	9	56	48
0.100	-	0	-	0	0	0	17	23
0.250	-	-	-	-	0	0	0	0
0.500	-	-	-	0	0	0	0	0
0.750	-	-	-	-	-	-	0	0
1.000	-	-	-	0	0	0	0	0
TL_{50}	0.001	0.005	0.005	0.010	0.025-0.05	0.025-0.05	0.075	0.075

TABLE 19

CORRELATION OF PERCENT EGG HATCHABILITY WITH
POLLUTION-INDUCED REDUCTION IN DIVERSITY OF PISCINE FAUNA

MONITORING SITE[1]	PERCENT EGG HATCHABILITY[2]			DIVERSITY OF FISH FAUNA[3]	
	FISH	AMPHIBIANS	FISH & AMPHIBIANS	# SPECIES/EXPECTED[4]	% SPECIES REMAINING
Cane Run	0	0	0	0/15	0
Town Branch	0	0	0	0/15	0
Wolf Run	40	18	24	2/15	13
East Hickman Creek	56	45	48	8/17	47
West Hickman Creek	63	58	60	11/16	69
Gainesway Branch	70	74	73	7/10	70
Hickman Creek	74	82	80	15/22	68
Shelby Branch	81	90	87	14/17	82
Kentucky River	83	89	87	21/25	84
Elkhorn Creek	86	92	89	43/48	90
Steele's Run	91	97	94	15/15	100

[1]Streams and rivers selected to represent various levels of water pollution resulting from recent increases in urbanization and industrialization in the Kentucky Bluegrass region.
[2]Egg hatchability data combined and averaged for four species of fish (rainbow trout, largemouth bass, goldfish, channel catfish) and five species of amphibians (squirrel treefrog, gray treefrog, narrow-mouthed toad, pig frog, red-spotted toad).
[3]Data on fish fauna for past 22 years taken from Carter (70), Jones (71), Kuehne (72), Laflin (73), MacGregor and Andre (74), Westerman and Westerman (75), and Small (76).
[4]Number species persisting at monitoring sites/number of species reported in earlier censuses.

APPENDIX II

SYNOPSIS OF BIOASSAY PROCEDURES

I. Preparation of Culture Water.

If an adequate supply of natural water free of background contamination is not available, reconstituted water may be used. A synthetic culture water which has been found satisfactory for bioassay procedures is given in Table 3, and possesses ionic composition and physicochemical characteristics similar to Mount's spring water (55). Synthetic culture water should be prepared from charcoal filtered, distilled-deionized or double deionized water having a conductivity of 0.25 μmhos or less. Routine monitoring should be conducted for possible background contaminants (e.g., heavy metals, chlorinated hydrocarbons).

Culture water may be prepared from the following three stock solutions. Reagent grade chemicals should be used.

1. $NaHCO_3$ 100 g/l
   KCl 5 g/l
2. $CaCl_2$ 60 g/l
3. $MgSO_4 \cdot 7H_2O$ 60 g/l

To prepare culture water with hardness levels of 50-200 ppm $CaCO_3$, stock solutions should be blended in the following ratios and diluted to a final volume of 20 liters using distilled-deionized water.

Stock Reagent	Water Hardness Level (ppm $CaCO_3$)		
	50	100	200
$NaHCO_3$ + KCl	20.0 ml	20 ml	20 ml
$CaCl_2$	12.5 ml	25 ml	50 ml
$MgSO_4 \cdot 7H_2O$	12.5 ml	25 ml	50 ml

II. Setup of Bioassay System.

Continuous flow bioassays should be set up and operating at least 24-72 hours prior to introducing test organisms, allowing sufficient time for adjustments and stabilization of toxicant level and other parameters (e.g., temperature, dissolved oxygen, pH). During this period all test parameters should be monitored at regular 8-12 hour intervals.

A. Preparation for testing.

Bioassays preferably should be conducted in walk-in, temperature-controlled environmental rooms, disinfected before use with a microbicide and 12-24 hours of ultra-violet irradiation. Bioassay glassware should be chemically cleaned using the procedure given below or some suitable alternative.

1. Rinse toxicant solutions from all glassware.
2. Scrub with Alconox detergent and rinse with tap water.
3. Rinse thoroughly in tap distilled water and allow to dry.
4. Soak in acetone for 15-20 minutes and allow to dry.
5. Rinse and soak in tap distilled water.
6. Soak in concentrated nitric acid for 15-30 minutes.
7. Rinse in glass-distilled water.
8. Soak for several hours in glass-distilled or deionized water.
9. Dry at 300^{o} F or autoclave.
10. Allow cooling to room temperature and cover with parafilm.
11. Store in closed dust-free cabinet.

B. Installation of peristaltic and syringe pumps.

Prior to performing bioassays, tests should be conducted on all pumping units to calibrate and evaluate reproducibility of flow rates. The variable speed Brinkmann (model 131900) and Gilson (models HP 8 and HP 16) peristaltic pumps, fitted with tygon tubing, generally give satisfactory performance. Periodic adjustments should be made in pumping rate to offset effects of wear on tubing. Tygon tubing should be replaced after 2-3 weeks of operation.

Syringe pumps should be equipped with up to six double-ground glass syringes. For this purpose we have used a model 355 Sage syringe pump equipped with a modified holder (Fig. 3). Generally, more satisfactory performance is obtained when syringe pumps are mounted outside the environmental room, obviating effects of reduced temperature and/or high humidity. Each set of syringes should be matched for equal diameter and stroke volume.

C. Setup of mixing and culture chambers.

Each culture chamber (e.g., 300 ml modified deep etri dish) is connected to a mixing chamber (e.g., 250 ml sidearm Erlenmeyer flask) using 1/4" i.d. polyethylene or teflon tubing. A separate peristaltic pump channel should be connected to each

mixing flask, using tygon tubing. A liquid flowmeter (size no. 12, Roger Gilmont Instruments, Great Neck, N.Y.) should be installed in each line to calibrate and monitor flow. Each mixing flask should receive its own syringe pump line. Teflon tubing (e.g., McMaster-Carr Co., Chicago, Ill.) should be used for this purpose, and a flowmeter (Gilmont Microflowmeter) should be inserted in each syringe pump line. Teflon rapid-disconnects may be used in connecting pumping lines to flow meters.

Culture water should receive continuous aeration supplied either to the mixing chamber or the culture water reservoir which feeds the peristaltic pump. The latter is advised when testing more volatile toxicants. The air line to the culture room should be equipped with a filter and moisture trap (e.g., McMaster-Carr Co., 40 μ or less).

D. Maintenance of syringe pumps.

While syringes may be removed individually from the pump and replaced with a fresh set, it is generally more expeditious to leave them mounted, retract the drive carriage and refill them in place. In either case, the syringe lines must be clamped securely to prevent toxicant discharge into the mixing chambers. Solutions used to fill syringes should be analyzed to confirm concentration of the toxicant to be delivered to the mixing flask. The rate of toxicant injection should be calculated to dilute culture water to desired exposure levels.

III. Acquisition and Care of Test Animals.

The investigator should be familiar with the basic biology of his selected test species, including some knowledge of (1) taxonomy, (2) geographical distribution, (3) ecology and natural history, (4) life cycle and reproductive behavior (e.g., optimum spawning temperature, hatching time), and (5) embryonic development (e.g., cleavage, gastrulation, organogenesis). Such background information is essential to the selection of test species and the use of developmental stages for bioassay purposes.

A. Selection of animal test species.

As noted above, we have found sensitivity to toxicants to vary substantially for eggs of different fish and amphibian species. Therefore, where possible, we recommend the use of 2-3 test species, selected for different developmental characteristics (e.g., egg type, yolk volume, hatching time, cleavage pattern). The fish and amphibian species we have used most extensively are listed in Table 10, together

with data on hatching time, egg production, seasonal availability and source of supply.

B. Obtaining gravid animals.

Gravid animals should be collected from sources reasonably free of environmental contaminants. Particular consideration should be given to chlorinated hydrocarbons and other toxicants with high bioaccumulation potential and which may concentrate to high levels in eggs and gonadal tissues, affecting hatchability. Federally supported investigators may coordinate through the Director of Sport Fisheries and Wildlife to obtain gravid animals from a number of Federal Hatcheries.

C. Maintenance of adult animals.

Once obtained, gravid animals may be maintained for short periods of time in large capacity non-flow, temperature-regulated fish tanks (e.g., 100 gal Living Stream fiberglass tank, Frigid Units, Toledo, Ohio). However, for longer storage, 150-300 gal flow-through, temperature-regulated, fiberglass tanks should be used. If an adequate supply of well or spring water is not available, charcoal filtered dechlorinated tap water generally proves satisfactory. The source of water should be monitored for possible background contaminants. If available water supply will not support an adequate flow-through culture, fish tanks may be equipped with high capacity recirculating filters (e.g., activated charcoal, filter floss). Such filters are particularly useful for removing food debris and other particulates from the culture water. Adequate aeration should be supplied to all fish tanks.

A high quality fish food should be used to support fish populations (e.g., Purina; Silver Cup). However, as certain lots of food may contain high levels of trace contaminants, it is advisable to analyze for suspected toxicants (e.g., heavy metals, chlorinated hydrocarbons).

D. Egg spawning techniques.

In the event freshly spawned eggs cannot be obtained in the laboratory or from local hatcheries, artificial spawning may be used. Animals should be selected in optimum spawning condition, as underripe or overripe eggs may exhibit substantially reduced hatchability (64, 65). The general appearance of the eggs and external features of the adult animal (e.g., body size and shape, breeding color, extension of the urogenital papilla) may be used as indicators of optimum spawning conditions. For best results, eggs maintained in control cultures should give at least 75% hatchability. Lower hatching frequencies generally indicate an inadequate source of eggs and sperm or faulty culture technique.

Egg and sperm collecting techniques vary with different test species. Using the method of Leitritz (64), the artificial stripping technique works well for the rainbow trout. Gravid females should be artificially spawned as soon as eggs can be obtained with a gentle to moderate stripping action. Males, which are usually ripe concurrent with the females, may be spawned several times during the breeding season. Eggs from the goldfish and numerous other species may be collected by similar procedures. Temperature change and/or hormonal injection (300 IU HCG/454 g body weight, administered I.P.) may be used to initiate spawning in such species as the goldfish. When artificial stripping is not feasible, eggs sometimes can be collected by the incision method (64). We have used the latter satisfactorily for both channel catfish and largemouth bass, despite a modest reduction in egg hatchability.

Once eggs and sperm have been collected, they should be mixed in spawning trays or large glass finger bowls for 10-15 minutes, avoiding exposure to fluorescent light. Preferably, eggs from several females should be mixed prior to setting up test sub-samples, to minimize individual variations in hatchability or sensitivity to toxicants.

It should be noted that eyed trout eggs (i.e., 12-15 developmental days) can be obtained by air express from a number of hatcheries and are particularly useful for initial bioassay screening studies. In addition, fresh catfish spawn can be successfully shipped by air express. It should be noted, however, that sensitivity of the bioassay may be somewhat diminished if early developmental stages are omitted from the test.

IV. Performance of Bioassay Tests.

A. Introduction of eggs to chambers.

Egg samples (100-150) should be introduced into each culture chamber, using a graduated beaker or measuring scoop. Final egg tallies may be delayed until all bioassay cultures have been established. Care should be taken not to overcrowd or unnecessarily contaminate cultures.

B. Monitoring of bioassay cultures.

Toxicant exposure levels should be confirmed by direct chemical analyses performed on all bioassay cultures at regular daily intervals. It is particularly important to stabilize toxicant concentration before introduction of eggs into the bioassay system, and to monitor exposure levels carefully during the first 24-48 hours after egg treatment is initiated.

Cultures also should be monitored daily for the following test parameters: temperature, pH, alkalinity, conductivity, dissolved oxygen, water hardness, and ammonia. Also, flow rates from both the syringe and peristaltic pumps should be measured at least twice daily.

C. Observation of test animals.

Test animals and culture conditions should be observed daily to gauge extent of development and remove dead specimens. Lack of embryonic development, discoloration of eggs and vascular degeneration are useful criteria for early recognition of embryonic mortality. Where necessary, reference may be made to standard embryological staging guides (64, 66-69).

Contamination of the cultures should be minimized by maintaining semi-sterile test conditions. In the event of soft egg disease or fungus, cultures may be treated according to the procedures recommended below:

1. Malachite green procedure.
   a. Prepare stock solution of malachite green by dissolving 100 g of dye in 100 ml distilled water.
   b. Dilute stock solution 1:15,000 (v/v) with distilled water.
   c. Decant the culture water from the exposure chamber.
   d. Add 100 ml of malachite green solution to the dish and let stand for 30 seconds.
   e. Decant malachite green solution.
   f. Rinse eggs carefully with fresh culture water to remove excess dye.
2. Betadine procedure.
   A 1:5 solution of Betadine (povidone-iodine, Purdue Frederick Co., Norwalk, Conn.) may be substituted for malachite green and used as given above.
3. Formaldehyde (1/1000 for 10 minutes) or acriflavine (1/2000 for 20 minutes) may be used as alternative methods of treatment.

As such treatment may introduce undesirable test variables, the most practical course may be to discard contaminated cultures and repeat the bioassay experiment.

APPENDIX III

NORMAL AND TERATOGENIC STAGES OF RAINBOW TROUT ALEVINS AND FRY

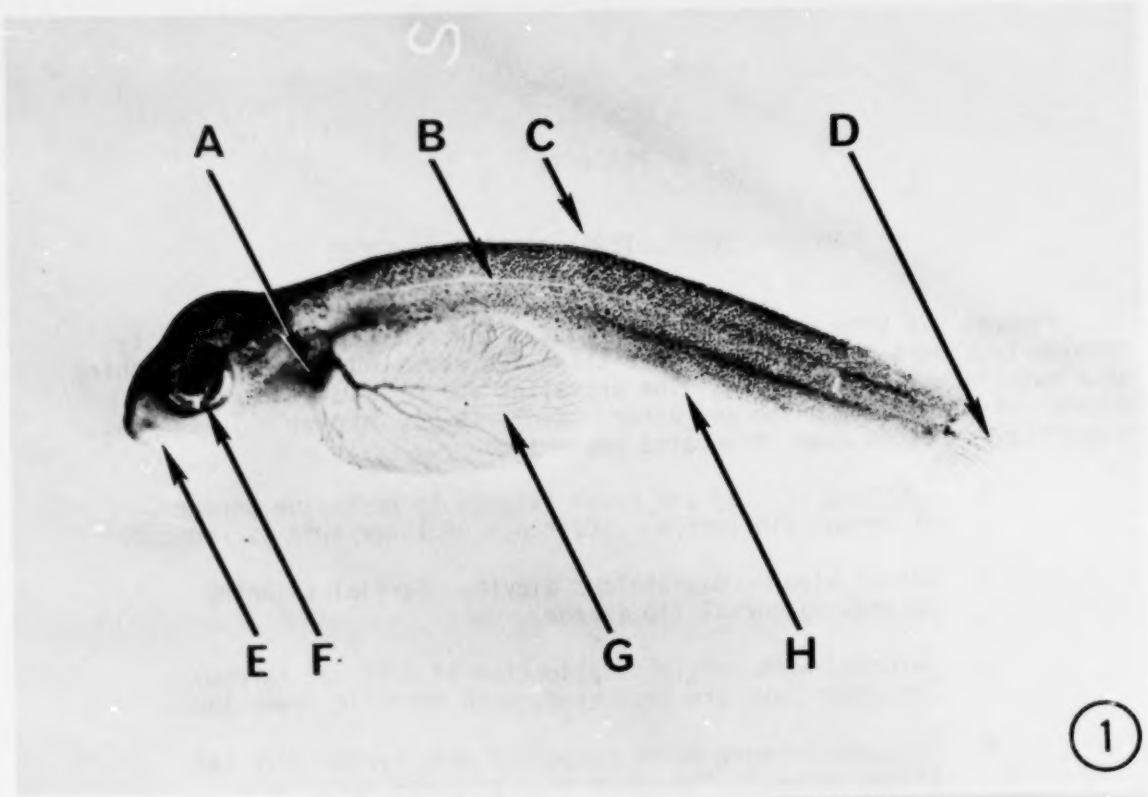
PLATES I-VI

PLATE I

NORMAL STAGES OF TROUT ALEVINS AND FRY

1. Trout alevin at 1 week posthatching, showing heart (A), lateral line (B), dorsal soft-ray fin (C), caudal fin (D), mandible (E), iris of eye (F), yolk sac with vitelline vessels (G), and urogenital papilla (H). 7.5X.

2. Normal trout fry at 21 days posthatching, showing external nares (A), operculum (B), dorsal soft-ray fin (C), adipose fin (D), caudal fin (E), mandible (F), pectoral fin (G), parr marks (H), pelvic fin (I), urogenital papilla (J), anal fin (K), and caudal peduncle (L). Parr marks represent a characteristic pattern of skin pigmentation appearing in juvenile stages at about 2 weeks. 10X.



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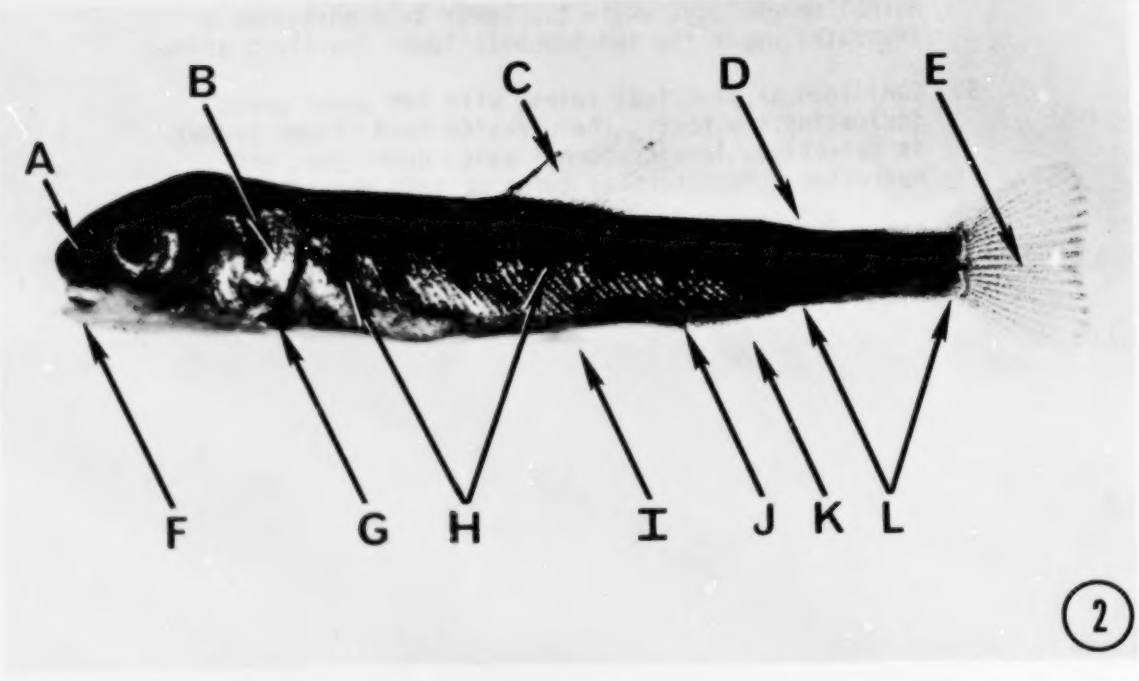

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PLATE II

CADMIUM-INDUCED TERATOGENESIS IN TROUT

Figures 1-6 show congenital deformities induced in trout embryos by cadmium treatment. Photographs were taken 1-2 weeks subsequent to hatching at a magnification of 7.5-10X. The anomalies shown illustrate various degrees of Siamese twinning and other defects (e.g., immovable lower jaw, anomalous or absent eyes, truncated upper jaw).

1. Twinning of head and trunk extends to posterior border of dorsal fin (arrow). Left eye of lower twin is reduced.

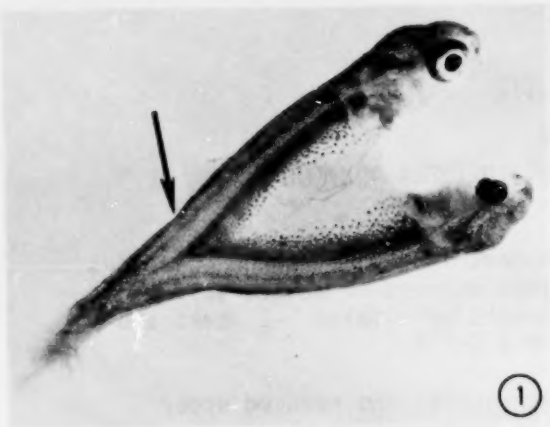
2. Dorsal view of dicephalous alevin. Partial twinning extends to dorsal fin (arrow).

3. Twinning with partial duplication of yolk sac (arrow). The upper jaws are truncated, with immobile lower jaws.

4. Complete Siamese twins conjoined only by the yolk sac (right arrow). The upper twin displays relatively normal morphology, while the lower twin possesses a truncated upper jaw and immobile lower jaw (left arrow).

5. Conjoined assymetrical twins, with the upper arrow indicating the host. The parasite head (lower arrow) is defective, lacking normal eyes, upper jaw, and operculum. Both partial twins possess dorsal fins.

6. A further example of partial twinning. The less developed head has defective eyes (arrow).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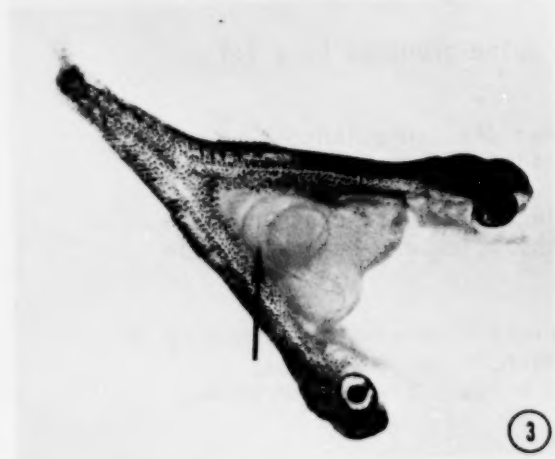
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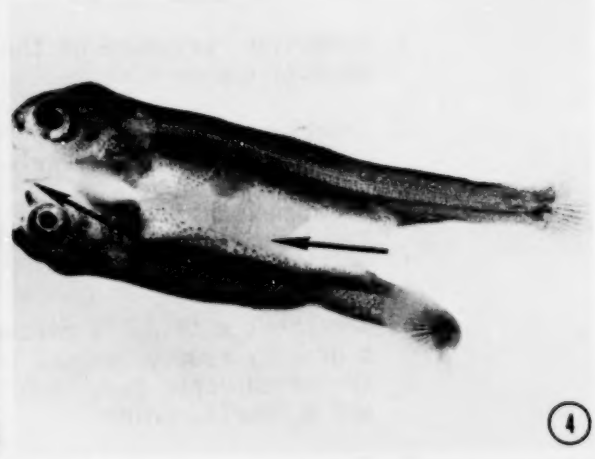
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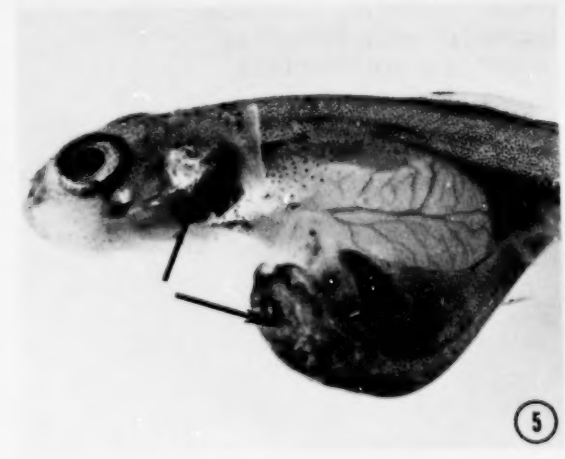
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PLATE III

TERATOGENIC DEVELOPMENT IN TROUT ALEVINS

Figures 1-6 show teratogenic development induced by boron compounds and heavy metals. Figures 1-4 show various degrees of acute, inflexible curvatures of the spinal column. Photographs were taken 1-2 weeks subsequent to hatching at a magnification of 7.5-10X.

1. Acute curvature of the spine (arrow) and reduced upper and lower jaws resulting from boron treatment.

2. Defective curvature of the spine produced by a 1:1 mercury-copper mixture.

3. Anomalous cephalic and spinal structure induced by exposure to a mercury-selenium mixture.

4. Cadmium-induced anomalies including a truncate upper jaw (arrow), curvature of the spine and defective iris of the eye.

5. Treatment with a 1:1 mercury-cadmium mixture resulting in a grossly reduced caudal peduncle (upper arrow), truncated upper jaw, immobile lower jaw (lower arrow) and a dwarfed trunk.

6. Boron-induced congenital defects including a reduced scoliotic caudal peduncle (upper arrow), defective eyes (lower arrow), absent upper jaw and immobile lower jaw.

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PLATE IV

TERATOGENIC DEVELOPMENT IN TROUT ALEVINS

Figures 1-6 show teratogenic defects produced by boron and heavy metals. Photographs were taken 1-2 weeks posthatching at a magnification of 7.5-10X.

1. Acute ventral flexion of spinal column induced by treatment with boron.

2. Acute lateral flexion of spinal column (scoliosis) produced by a mercury-copper mixture.

3. Cadmium-induced flexion of the spinal column.

4. Acute, inflexible curvature and irregular structure of spinal column resulting from treatment with cadmium.

5. Kyphosis of the spine (arrow) and dwarfed trunk resulting from an exposure to a mercury-selenium mixture.

6. Anomalous spinal column and dwarfed trunk (arrow) produced by treatment with a mercury-copper mixture.

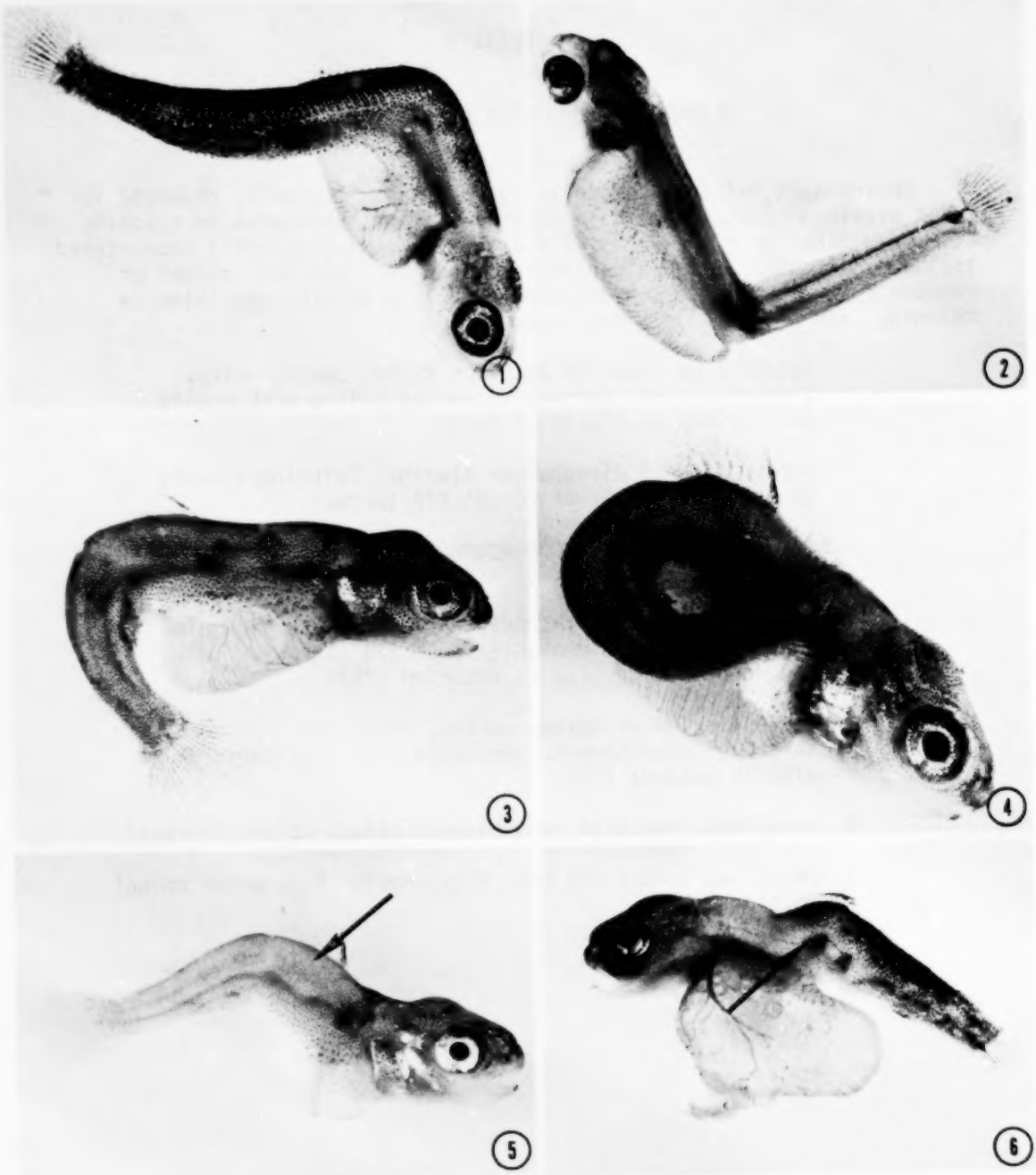
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PLATE V

MERCURY-INDUCED TERATOGENESIS IN TROUT

Photographs 1-7 show congenital deformities frequently observed in trout alevins. Photographs were taken 1-2 weeks subsequent to hatching at a magnification of 7.5-10X. The anomalies most frequently encountered included defective vertebral columns, immovable lower jaw, absent or reduced fins, absent eyes, hydrocephalous brain development, Siamese twinning, and retarded yolk sac reabsorption.

1. Twinning of head and anterior trunk, ventral view. Single yolk sac (arrow) possesses paired yolk stalks, one serving each partial twin.

2. Dorsal view of dicephalous alevin. Twinning extends to anterior border of dorsal fin (arrow).

3. Inflexible C-shaped curvature of tail, accompanied by hydrocephaly (arrow).

4. Skeletal anomalies including an acute lordotic spine and a defective, immovable lower jaw (arrow). Yolk sac reabsorption also is retarded (YS).

5. Acute flexion of spinal column, resulting from defective vertebral development. Retarded yolk sac reabsorption also is evident (YS).

6. Anomalous, immobile curvature of spinal column (arrows).

7. Defective dorsal fin and sharp angular flexion of spinal column.

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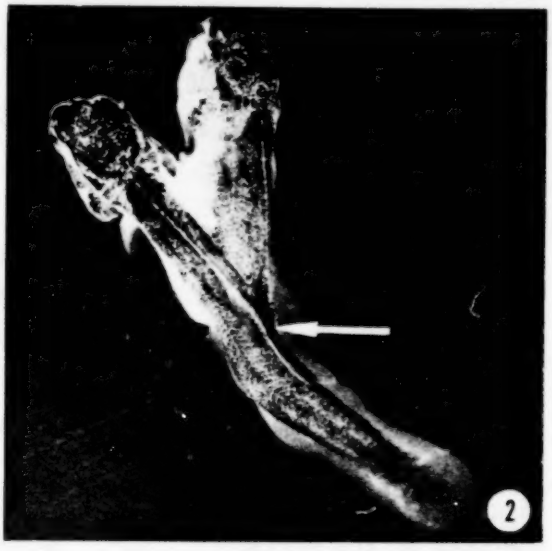

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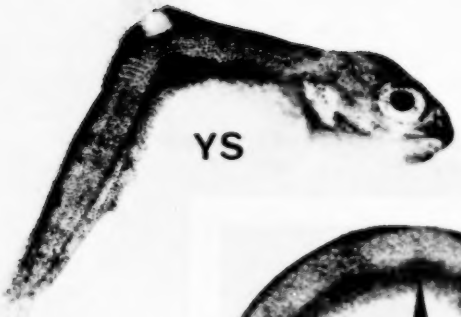

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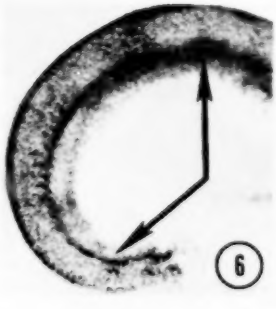

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PLATE VI

BORON-INDUCED TERATOGENESIS IN TROUT

Photographs 1-6 show congenital deformities frequently observed in trout alevins which survived treatment with boron compounds. Photographs were taken 1-2 weeks subsequent to hatching at a magnification of 7.5-10X. The anomalies most frequently encountered included various acute defects of the vertebral column, amphiarthrotic (immobile) jaw articulation, absent or reduced fins, anomalous or absent eyes, dwarfed or irregular trunk morphology, Siamese twinning, and retarded yolk sac resorption.

1. Skeletal anomalies including an acute, lordotic spine, attenuated tail fin, and a defective, immobile lower jaw (arrow).

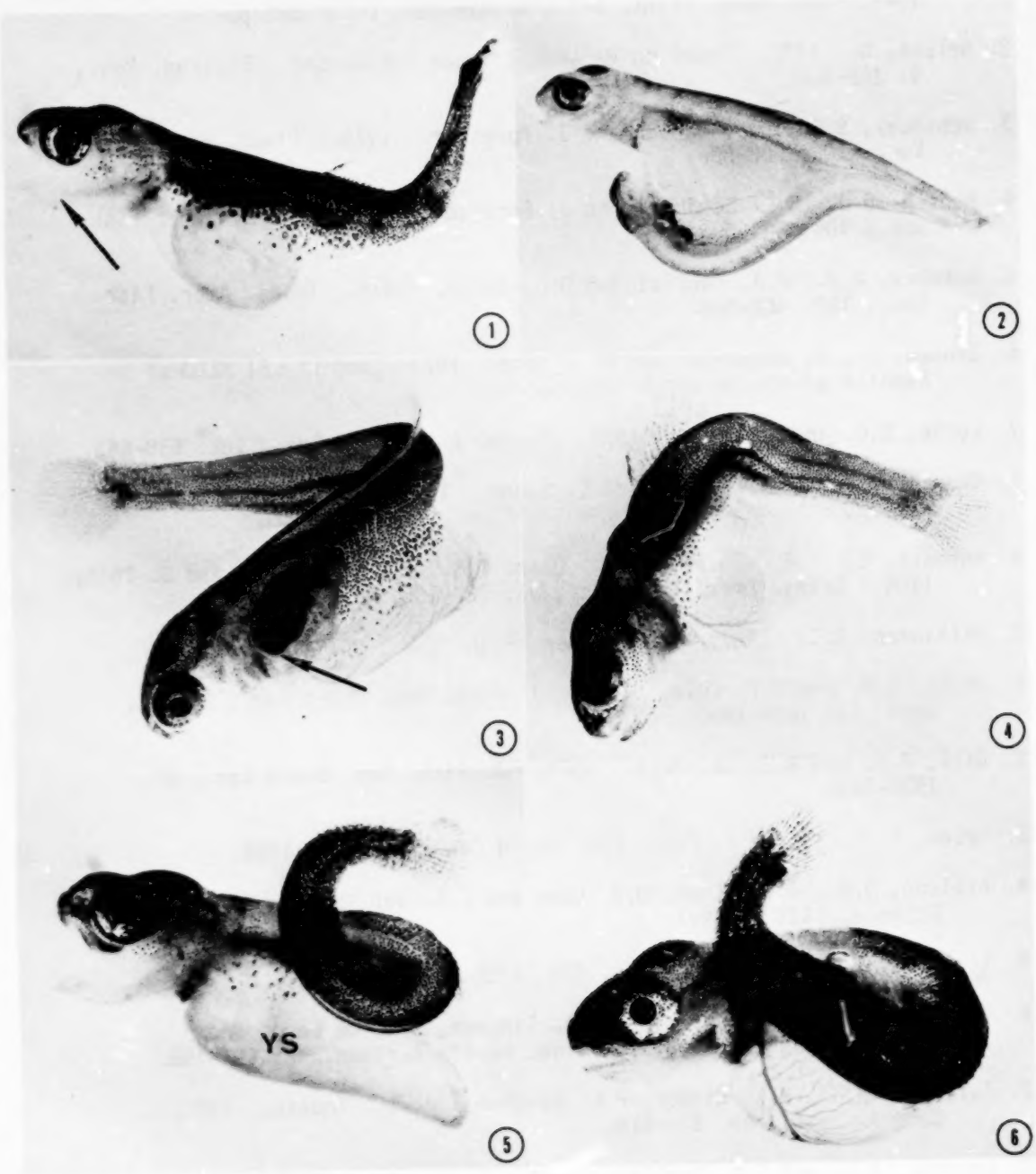
2. Twinning of head and trunk, with a single tail. A single yolk sac possesses paired yolk stalks, one serving each partial twin.

3. Acute scoliosis of the vertebral column and cephalic twinning. Secondary head (arrow) is only partially formed.

4. Kyphosis of the vertebral column with irregular, dwarfed trunk structure.

5. Anomalous, inflexible curvature of spinal column, irregular, truncate upper jaw, immobile lower jaw, and retarded yolk sac resorption. Also, as seen in Figures 1 and 6, incomplete closure of the choroid fissure has resulted in coloboma of the retina. This condition is discernible as a vertical cleft at the mid-inferior border of the eye.

6. Irregular, fixed curvature of the vertebral column similar to that shown in Figure 5.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BIBLIOGRAPHY

1. NAS-NAE Committee on Water Quality Criteria. 1972. Water Quality Criteria 1972. U.S. Govt. Print. Off., Washington, D.C. 593 pp.

2. Nelson, N. 1972. Panel on Hazardous Trace Substances. Environ. Res., 5: 249-362.

3. Schimmel, S.C., D.J. Hansen and J. Forester. 1974. Trans. Amer. Fish. Soc., 103: 582-586.

4. Hansen, D.J., S.C. Schimmel and J. Forester. 1975. Trans. Amer. Fish. Soc., 104: 584-588.

5. Nebeker, A.V., F.A. Puglisi and D.L. DeFoe. 1974. Trans. Amer. Fish. Soc., 103: 562-568.

6. Jensen, S., N. Johansson and M. Olsson. 1970. Report LFI MEDD of the Swedish Salmon Research Institute.

7. Locke, D.O. and K. Havey. 1972. Trans. Amer. Fish Soc., 101: 638-643.

8. Cuerrier, J.P., J.A. Keith and E. Stone. 1967. Naturaliste Can., 94: 315-320.

9. Burdick, G.E., E.J. Harris, H.J. Dean, T.M. Walker, J. Skea and D. Colby. 1964. Trans. Amer. Fish Soc., 93: 127-135.

10. Hiltibran, R.C. 1967. Trans. Amer. Fish. Soc., 96: 414-416.

11. Smith, R.M. and C.F. Cole. 1975. J. Fish. Res. Board Can., 30 (12, part 1): 1894-1898.

12. Dill, P.A. and R.C. Saunders. 1974. J. Fish. Res. Board Can., 31: 1936-1938.

13. Macek, K.J. 1968. J. Fish. Res. Board Can., 25: 1787-1796.

14. Allison, D.B., J. Kallman, O.B. Cope and C.C. Van Valin. 1963. Science, 142: 958-961.

15. Sterling, T.D. 1971. Science, 174: 1358-1359.

16. Bowes, G.W., B.R. Simoneit, A.L. Burlingame, B.W. de Lappe and R.W. Risebrough. 1973. Environ. Health Perspec., 2: 191-198.

17. Aulerich, R.J., R.K. Ringer, H.L. Seagran and W.G. Youatt. 1971. Can. J. Zool., 49: 611-616.

18. Nebeker, A.V. and F.A. Puglisi. 1974. Trans. Amer. Fish. Soc., 103: 722-728.

19. Hansen, D.J., P.R. Parrish, J.I. Lowe, A.J. Wilson, Jr. and P.D. Wilson. 1971. Bull. Environ. Contam. Toxicol., 6(2): 113-119.

20. Johnson, D.W. 1968. Trans. Amer. Fish. Socl., 97: 398-424.

21. Johnson, H.E. and C. Pecor. 1969. Trans. N. Amer. Wildl. Natur. Resour. Conf., 34: 159-166.

22. Lane, C.E. and R.E. Livingston. 1970. Trans. Amer. Fish. Soc., 99: 489-495.

23. Johnson, H.E. 1967. The Effects of Endrin on the Reproduction of a Fresh Water Fish (Oryzias latipes). Ph.D. dissertation, University of Washington, Seattle. 149 pp.

24. Birge, W.J., A.G. Westerman and O.W. Roberts. 1974. In 2nd Annual NSF-RANN Trace Contaminants Conference, Trace Contaminants in the Environment, 3: 316-320.

25. Pickering, Q.H. and W.N. Vigor. 1965. Progve. Fish Cult., 27: 153-157.

26. Skidmore, J.F. 1965. J. Fish. Res. Board Can., 23: 1037-1041.

27. _____. 1965. Ann. Appl. Biol., 56: 47-53.

28. Brungs, W.A. 1969. Trans. Amer. Fish. Soc., 98: 272-279.

29. Hazel, G.R. and S.J. Meith. 1970. Calif. Fish Game, 56: 121-124.

30. McKim, J.M., G. Christensen and E. Hunt. 1970. J. Fish. Res. Board Can., 27: 1883-1889.

31. Birge, W.J. and J.J. Just. 1975. U.S. Dept. Interior, Res. Report #84. 36 pp.

32. _____ and J.J. Just. 1974. U.S. Dept. Interior, Res. Report #71. 33 pp.

33. _____ and O.W. Roberts. 1976. Bull. Environ. Contam. Toxicol., 16(3): 319-324.

34. _____, O.W. Roberts and J.A. Black. 1976. Bull. Environ. Contam. Toxicol., 16(3): 314-318.

35. Wallen, I.E., W.C. Greer and R. Lasater. 1957. Sew. Ind. Waste, 29: 695.

36. Turnbull, H., J.G. DeMann and R.F. Weston. 1954. Symposium on Waste Disposal in the Petroleum Industry, Ind. Eng. Chem., 46: 324.

37. Sprague, R.W. 1972. The Ecological Significance of Boron. U.S. Borax Research Corporation, Anaheim, Calif. 58 pp.

38. Alabaster, J.S. 1957. Proc. Brit. Weed Contr. Conf., 3: 807.

39. Birge, W.J., J.A. Black, A.G. Westerman, J.E. Hudson and R.A. Freeman. 1976. Lehtal and Teratogenic Effects of Metallic Pollutants on Embryogenesis and use of Vertebrate Embryos as Sensitive Indicators of Environmental Quality. Completion Report, NSF (RANN).

40. Takeuchi, T. 1958. Sci., Rep. Tohoku Univ. Ser. 4, Biol. 24 (1): 33-43.

41. Hermanni, H.H. 1972. Wilhelm Roux' Archiv., 171: 200-222.

42. Goldie, M. and H. Stierholz. 1964. J. Amer. Osteo. Assoc., 63 (9): 879-880.

43. Landauer, W. 1952. J. Exptl. Zool., 120 (3): 469-507.

44. _____. 1953. Genetics, 38: 216-228.

45. Birge, W.J. and J.A. Black. 1976. Sensitivity of Vertebrate Embryos to Boron Compounds. Completion Report, EPA. pp. 1-62.

46. Hove, E., C.A. Elvehjem and E.B. Hart. 1939. Amer. J. Physiol., 127: 689-701.

47. National Technical Advisory Committee on Water Quality Criteria, U.S. Dept. Interior. 1968. 234 pp.

48. Hynes, H.B.N. 1960. The Biology of Polluted Waters. Liverpool University Press, Liverpool. 202 pp.

49. Tarzwell, C.M. 1971. Proc. Roy. Soc. Lond. B., 177: 279-285.

50. Warren, C.E. 1971. Biology and Water Pollution Control. W.B. Saunders Co., Philadelphia. 434 pp.

51. Birge, W.J., A.G. Westerman and J.A. Black. 1975. U.S. Dept. Interior, Res. Report #91. 27 pp.

52. Waller, W.T. and J. Cairns, Jr. 1972. Wat. Res., 6: 257-269.

53. Sparks, R.E., J. Cairns, Jr., and A.G. Heath. 1972. Wat. Res., 6: 895-911.

54. LeClerc, E. and F. Devlaminck. 1955. Bull. de Belge. Condument Eaux., 28: 11.

55. Mount, D.I. 1968. Wat. Res., 2(3): 215-223.

56. Dutt, G.R. and T.W. McCreary. 1970. Univ. Arizona Ag. Exper. Station, Report #256. 83 pp.

57. Water Resources Data for Kentucky, Part 2. 1970. U.S. Dept. Interior Geol. Survey. 173 pp.

58. McKee, J.E. and H.W. Wolf. 1963. Water Quality Criteria. The Resource Agency of Calif. (2nd ed.). State Water Quality Control Board, Publ. #3A. 548 pp.

59. Hart, W.B., P. Doudoroff, and J. Greenbank. 1945. Evaluation of Toxicity of Industrial Wastes, Chemicals and Other Substances to Freshwater Fishes. Water Control Lab., Atlantic Refining Co., Philadelphia.

60. Bass Becking, L.G.M., I.R. Kaplan and D. Moore. 1960. J. Geol., 68: 243-284.

61. MacCrimmon, H.R. and W.H. Kwain. 1969. Can. J. Zool., 47: 631-637.

62. Eisler, R. 1957. Trans. Amer. Fish. Soc., 87: 151-162.

63. Roberts, R.F. and H.E. Allen. 1972. Trans. Amer. Fish. Soc., 101 (4): 752-756.

64. Leitritz, E. 1959. Trout and Salmon Culture. State of California, Department of Fish and Game. Fish Bulletin No. 107, 169pp.

65. Fish, G.R. and G.D. Ginnelly. 1966. Trans. Am. Fish. Soc., 95: 104-107.

66. Rugh, R. 1962. Experimental Embryological Techniques and Procedures. 3rd Ed. Burgess Publishing Co., Minneapolis, Minn. 501pp.

67. Knight, A.E. 1963. Trans. Am. Fish. Soc., 92: 344-355.

68. Volpe, E.P., M.A. Wilkens, and J.L. Dobie. 1961. Copeia (No. 3): 340-349.

69. Blaylock, B.G. and N.A. Griffith. 1971. Prog. Fish-Cult., 33: 48.

70. Carter, B.T. 1954. Trans. Ky. Acad. Sci., 15(3): 48-56.

71. Jones, A.R. 1973. Ky. Fish. Bull., No. 56. 108 pp.

72. Kuehne, R.A. 1975. U.S. Dept. Interior, Research Report #85. 33 pp.

73. Laflin, B.D. 1970. Ky. Fish. Bull., No. 55. 46 pp.

74. MacGregor, J.R. and C.A. Andre. 1975. Environmental Impact Statement for Lexington, Kentucky. Unpublished Report.

75. Westerman, A.G. and D.J. Westerman. 1975. Unpublished data.

76. Small, J., Jr. 1975. Ecology, 56 (4): 827-840.